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Quantitation of yohimbine utilizing capillary electrophoresis. Method development through statistical design.

Jonathan Cooper

Quantitation of Yohimbine Utilizing Capillary Electrophoresis. Method Development through Statistical Design.

Jonathan W. Cooper

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Thesis

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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QUANTITATION OF YOHIMBINE UTILIZING CAPILLARY ELECTROPHORESIS. METHOD DEVELOPMENT THROUGH STATISTICAL DESIGN.

Date: 12/1/99 Signature: _____

Dedicated to the memory of Dr. John E. Kraseski D.M.D.

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ACKNOWLEDGEMENTS.

I would like to thank my advisor, Dr. Paul Craig, without whom this research would not have been possible. His continuing support and suggestion were essential towards driving this project to completion, and his faith in my abilities allowed me to begin to discover and develop my own professional interests. Many thanks to Dr. Carol Marchetti for her contributions to the statistical aspects of this study. Her enthusiasm and motivation helped to turn a seemingly imposing statistical tool into an interesting and beneficial challenge, one that I hope others will follow my footsteps toward. I would also like to extend my gratitude to James Wesley for helping direct the path of this investigation, and Dr. T. C. Morrill for his guidance during my graduate career. I thank the Rochester Institute of Technology Department of Chemistry for giving me the opportunity to earn this degree, as well as providing me with a solid educational foundation from which I can expand.

I thank my family for their unconditional love and understanding. These past few years have been challenging for all of us in ways other than academic, but somehow we have been able to maintain and even develop our unique relationships. As I complete this project and look toward the future, there are a large number of uncertainties that I will soon have to face. Fortunately for me, my family will never be one of them.

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ABSTRACT.

In an effort to combat the rising cost of health care in the United States by encouraging preventative and self-care, the federal government passed the Dietary Supplement Health Education Act of 1994. This act essentially deregulated the natural product market allowing herbs, including yohimbine, to be sold over the counter without undergoing the same strict evaluation as a traditional synthetic drug candidate. As the full potential of these natural products is realized, including possible harmful side effects, a need arises for the identification and quantitation of the active components from commercial preparations.

Commercial samples were qualitatively and quantitatively analyzed for yohimbine utilizing a capillary electrophoretic method optimized through statistical design. Calibration curves produced with this method routinely achieved $R^2 > 0.998$. Method reproducibility (n = 10) and extraction efficiency (99.8% recovery) were determined to be sufficient. It was shown that a statistical design could limit the amount of experimental runs while providing a significant amount of information about experimental parameters.

INTRODUCTION.

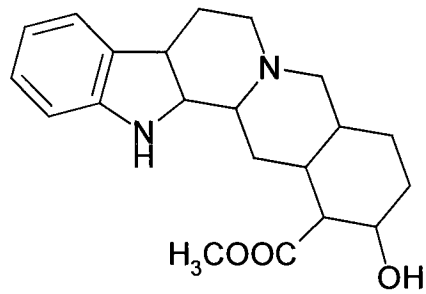
Yohimbine Background.

The West African tree Yohimbe is located in the low altitude, jungle forests enveloping southwestern Nigeria. Growing from 20 to 50 feet high, this tall evergreen forest tree has glabrous, leathery leaves three to five inches in length with upcurving lateral nerves that fade out at the margins. White flowers, containing winged seeds, are arranged in umbel-like clusters at the ends of the shoots (1, 2). It is the compounds found in the bark of this tree that have historically drawn attention towards the Yohimbe.

Documentation of the Bantu-speaking people describes yohimbe as an aphrodisiac and stimulant traditionally used during mating rituals that last up to 15 days. There are several methods of preparing yohimbe bark for use (2). A tea is prepared by boiling yohimbe bark shavings in water for less than four minutes. Heat is then turned down, allowing the brew to simmer for an additional 20 minutes. The shavings are strained, and the tea is sipped one hour before the desired effects are to be realized.

A method that leads to the effects in a shorter time after ingestion involves extraction of the bark powder with a drinkable alcohol. After a period of eight hours, the alcohol is strained and evaporated, leaving a residue that can be snuffed or taken subcutaneously. Effects through this method are more pronounced, occurring within twenty minutes (2).

The bark is reported to contain up to 6% yohimbine (Figure 1), along with a number of similar indole alkaloids including yohimbenine, isoyohimbine (mesoyohimbine), dihydroyohimbine, ajmalicine, and corynantheine (1). Yohimbine in



Yohimbine

Figure 1. Molecular structure of Yohimbine.

the form of the hydrochloride is also known as quebrachine, isolated from the South American evergreen Quebracho tree (2, 3) as well as from *Rauwolfia viridis* leaves (4), *Rauwolfia caffra* root bark and seeds (5), *Rauwolfia serpentina* root (6), *Rauwolfia oreogiton* root (7), *Rauwolfia volkensii* root (7), *Rauwolfia vomitoria* root (8), and *Rauwolfia yunnanensis* (9).

Yohimbine is the most interesting alkaloid from these sources, having gained notoriety in a number of circles as an alternative medicine treatment. Suggested for human use in bodybuilding as an alternative to anabolic steroids (1, 10), yohimbine has been employed in veterinary medicine as an antagonist for anesthesia and sedation of animals, as well as for the treatment of impotent stallions (11, 12). Classified as an alpha-2-adrenergic blocker, this sympathomimetic indole-type alkaloid also displays serotonin inhibiting properties (1, 2). In both humans and animals, yohimbine produces a complex pattern of responses that include anti-diuresis and central excitation, comprising elevation of blood pressure and heart rate, increased motor activity and irritability (13). Recent focus has been concerned with the aphrodisiac properties and use of yohimbine as a treatment for human erectile dysfunction (14-35).

The marketing of yohimbine falls under the Dietary Supplemental Health Education Act (DSHEA) of 1994, a governmental effort to limit the skyrocketing cost of United States health care that has essentially deregulated the natural product market. Natural products are not strictly evaluated pre-market as a synthesized drug candidate would be, and tend to draw scrutiny only when adverse effects are noted as a result of consumer use. Regulatory action toward another natural product with central nervous system stimulant properties, ephedrine, arose only after toxic levels were witnessed clinically (36, 37). These complications may be due to the inherent properties of the sources from which these natural products are harvested, namely vegetation. Alkaloid quantities from two distinct plants may be different from different regions, quantities may be different between two distinct plants in the same region, or quantities may be different between two leaves on the same tree. A preparation of natural products may in turn have varying concentrations between batches, between bottles, and even between pills in the same bottle for this reason. This is an important consideration for a compound that has a small range between a therapeutic dose and toxic dose, when product variance could mean the difference between life and death.

As the drawbacks of the DSHEA are eventually realized and attention focuses on dose reproducibility, qualitative and quantitative characterization of many natural products, including yohimbine, will gain importance. Efforts have thus far been reported toward the identification and quantification of yohimbine in commercial products and biological fluids utilizing fluorescence (6), ultra-violet spectroscopy (UV) (5-8), infrared spectroscopy (IR) (7, 8), nuclear magnetic resonance spectroscopy (NMR) (8), thin-layer chromatography (TLC) (6, 38, 39), gas chromatography (GC) (10), gas chromatography/

mass spectroscopy (GC/MS) (8, 13), gas-liquid chromatography (GLC) (5), liquid chromatography (LC) (40), and high-performance liquid chromatography (HPLC) (6, 13, 39-44). There have been no reported investigations of yohimbine utilizing capillary electrophoresis, a technique that is swiftly gaining prominence in the analytical laboratory.

History of Capillary Electrophoresis.

In the 1930's Arne Tiselius developed the first moving boundary electrophoresis method utilizing an open U-shaped quartz column. Protein mixtures in free solution were partially resolved as abutting bands detected by ultraviolet absorbance (45). However, this pioneering technique had drawbacks that included large sample volumes, and an incomplete separation of the proteins.

Investigation of improved anticonvective media in subsequent decades directed the focus toward zone electrophoresis. Polyacrylamide gels, developed in the late 1960's, contain stacking and resolving buffer systems that yield native protein and sodium dodecyl sulfate (SDS) bound protein separations (46). Known as polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, these techniques continue to give high-resolution separations as important tools in biochemical characterization.

Around the time PAGE was designed to separate proteins on the basis of charge or size, an investigation was underway to develop a separation based on protein isoelectric points. Isoelectric focusing (IEF) employs a gel containing an immobilized pH gradient constituted of a mixture of ampholytes. Vesterberg is credited with

deprotonated (50). Hydrated buffer cations coordinate to the anions at the capillary wall to form the rigid Helmholtz layer, and the mobile Gouey-Chapman layer to complete electroneutrality (Figure 3). A drop in negative potential, described by the zeta potential, characterizes the Gouey-Chapman layer: the magnitude of wall negative charge is directly proportional to layer thickness. While this region extends only a few nanometers beyond the Helmholtz layer, it is the mobile cations contained within that generate bulk electroosmotic flow. Mobile hydrated cations outnumber mobile anions in this area,

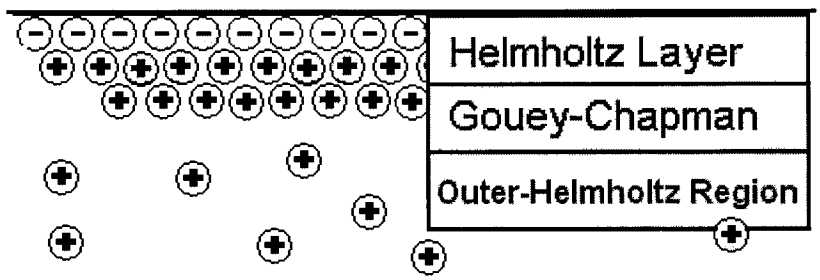


Figure 3. Interactions at the capillary wall that fuel electroosmotic flow. Not shown: hydrated anions in the diffuse layer (Outer-Helmholtz region).

resulting in a net flow toward the cathode under an applied voltage. Immediately upon the application of voltage, cations in this layer will have a greater velocity then the remainder of the capillary cross section. The resultant flow profile may have a parabolic character as the electrophoretic mobility of cations in the diffuse portion of the capillary are partially shielded by coordinated mobile anions with an opposite μ_e. This phenomenon would appear similar to a laminar flow profile towards the anode, but in fact EOF is in the direction of the cathode (Figure 4). Once a steady state is reached, a 'plug-

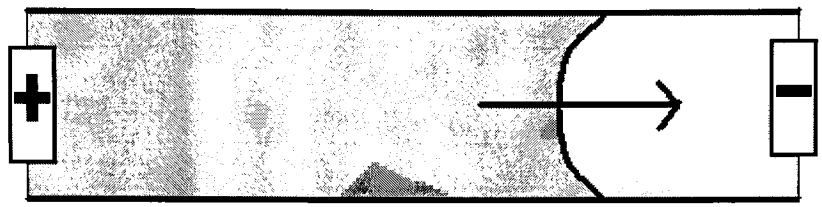


Figure 4. Reverse laminar flow diagram

like' flow is obtained at any cross sectional area beyond the Helmholtz plane, as this pulling force is uniform for the length of the capillary. Cation flow near the capillary wall inside the rigid Helmholtz layer approaches zero, resulting in the accepted depiction of electrically driven flow (Figure 5).

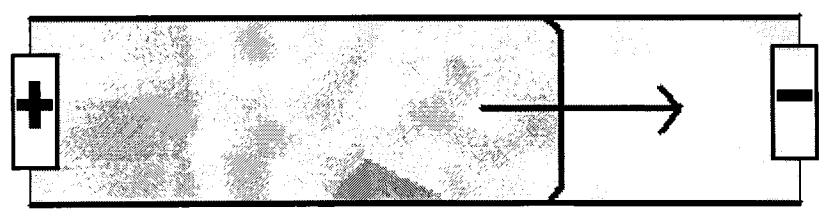


Figure 5. 'Plug-like' electroosmotic flow

Modes of Capillary Electrophoresis.

The term capillary electrophoresis describes the family of separation techniques based on the mobility differences of charged species under an applied voltage. Mention should be given to the variations on this empirical analytical technique that have gained

popularity toward specialized analysis. A summary of the notable techniques and applications follows.

Capillary Zone Electrophoresis (CZE).

Capillary zone electrophoresis is the electrophoretic separation of ions in a buffer solution under an applied voltage.

Micellar Kinetic Capillary Chromatography (MEKC).

Upon the addition of anionic surfactant molecules above a critical micelle concentration (CMC), hydrophobic tails of these molecules coordinate, exposing their hydrophilic heads and assuming a spherical shape characteristic of a micelle. These micelles have a μ_e toward the anode. Analytes in this electrophoretic system are further resolved on the basis of hydrophobicity as interaction with the micelles delays the observed migration.

Capillary Gel Electrophoresis (CGE).

The capillary is loaded with a gel, such as polyacrylamide, commonly employed in PAGE. Analytes are then separated based on charge to size ratio under an applied voltage. This technique has a high resolving power for proteins, peptides, and amino acids.

Capillary Isoelectric Focusing (CIEF).

An electrolyte containing a mixture of ampholytes and sample is loaded onto the column. When voltage is applied, the ampholytes migrate to form a continuous pH gradient in the capillary. Analytes migrate to the location on capillary that corresponds to their pI. The voltage is removed, and capillary contents are eluted past the detector under an applied pressure.

Capillary Isotachophoresis (CITP).

This is a moving boundary electrophoretic technique. The sample plug is introduced after a leading electrolyte with mobility faster than the fastest analyte, and before a terminating electrolyte of slower mobility than the slowest analyte. Compounds are resolved within the sample plug as it migrates toward the detection window.

Capillary Electrochromatography (CEC).

CEC is a capillary electrophoretic technique utilizing a capillary packed with one of any number of stationary phases used in HPLC. This technique can employ pressure, voltage, or a combination of the two as the driving separation force. This is useful in the separation of charged analytes that differ in hydrophobicity. The investigation of this technique has aided in the advancement of micro-HPLC systems, as pump technology advances.

Factorial Experimental Design.

Recently, statistical experimental designs have been increasingly employed as a useful option in analytical method development. A factorial design is equipped to address several major experimental concerns for which the traditional One Factor at a Time (OFAAT) trial and error development technique is not suited (52).

Joint effects or interactions are the effect that the simultaneous variation of two or more experimental parameters will have on response. These effects can only be identified and studied by the correlative change of the selected factors. The OFAAT method involves systematically optimizing one experimental variable, while holding all other variables constant. The method is then optimized for a second variable, again keeping all other variables constant. Subsequent variables are investigated in the same manner. To evaluate joint effects with this design, it is necessary to reevaluate variable one upon optimization of variable two, then the reevaluation of variable two with changes in variable one, eventually moving through all variables in this systematic approach. Clearly, the number of experimental runs would quickly become exhausting as the number of investigated variables increased.

A factorial experimental design allows for the systematic investigation of joint effects by enabling a statistical analysis to interpret the significance of factor levels, while limiting the number of experimental runs needed. This design is useful when expense, quantity of material or time are limited, as experiment numbers are significantly reduced.

There are several software programs that are capable of handling the complex calculations to analyze data from a factorial design, but it is useful to understand how these values are computed to employ this technique.

development of the method for synthesizing ampholyte mixtures for high-resolution IEF in 1969, still a separation technique found in the modern biochemistry laboratory (47).

O'Farrell combined IEF with SDS-PAGE in the 1970's, creating two-dimensional electrophoresis, a technique capable of resolving more than 50 components in each direction. Utilizing an isoelectric point separation followed by an electrophoretic separation in the perpendicular direction, several thousand compounds can be isolated with this approach (48).

During the late 1970's, the family of gel electrophoretic techniques gained acceptance in biochemistry laboratories, opening doors for investigation of protein-based biopharmaceuticals. Meanwhile, high performance liquid chromatography (HPLC) was gaining notoriety as an analytical tool in pharmaceutical and chemical laboratories. Along with high resolution, HPLC offered ease of automation, and excellent quantitative precision for the determination of low molecular weight compounds and industrial polymers (49). As gel techniques were applied in an industrial setting, their detection limitations and inherent lengthy, involved preparation procedures did not compare favorably to the swift and reliable chromatographic techniques employed at the time. This prompted pharmaceutical and bioanalytical chemists in these fields to turn their attention to HPLC for biomolecule characterization as protein, peptide, and nucleic acid chromatographic media became available during the early 1980's, (50).

A strong environmental awareness movement occurred in the 1980's as the biotechnology field broadened. This involved a chain of events effectively increasing waste disposal costs. A main analytical tool at this time, HPLC, routinely generates large amounts of organic solvent waste, which inferred a high cost of daily operation based on

Consider the case of a full factorial $2^k = 2^3$ design: variation of three factors (k = 3) at two levels. For example; the effect of temperature (factor A: 25°, 37°), pressure (factor B: 1 atm, 3 atm), and wavelength (factor C: 300 nm, 500 nm) on photosynthesis by a newly discovered algae. An experimental run chart is assembled and completed, recording the response (Table 1). There are four sets of experimental runs where factor

Table 1. 2^3 Factorial experimental design. Three factors (A,B,C) at two levels: low (-) and high (+).

Run Number	Factor A	Factor B	Factor C	Response (X)
1	-	-	-	X_1
2	+	-	-	X_2
3	-	+	-	X_3
4	+	+	-	X_4
5	-	-	+	X_5
6	+	-	+	X_6
7	-	+	+	X_7
8	+	+	+	X_8

A is varied as factors B and C remained constant. Runs 1 and 2, where A changes while B and C are both negative: Runs 3 and 4, where B is positive, and C is negative: Runs 5 and 6, where B is negative and C is positive: Runs 7 and 8, where B and C are both positive. Therefore, the effect of factor A can be described through Equation 2.

$$\text{Effect A} = \frac{((X_2-X_1) + (X_4-X_3) + (X_6-X_5) + (X_8-X_7))}{4} \qquad 2$$

The number generated describes the effect of moving from a low to a high level in A, regardless of the levels of B and C. This calculation can be completed for each of the factors employed in a factorial design to determine their effect (52).

Joint effects are handled in a similar manner. For this analysis, the design has two pairs of data points with a variation of A; one at the high level of B, the other at the low level, while C remains constant. Half of the difference of these two pairs is the value of the AB interaction (Equation 3).

$$\text{Effect A at positive B} = \frac{((X_8 - X_7) + (X_6 - X_5))}{2}$$

$$\text{Effect A at negative B} = \frac{((X_4 - X_3) + (X_2 - X_1))}{2}$$

$$\text{Effect AB} = \frac{((\text{Effect A at positive B}) - (\text{Effect A at negative B}))}{2} \qquad 3$$

Scheme 1.

This calculation can be completed for each factor of interest to determine the magnitude of joint effects. Utilizing computer software in the handling of these equations dramatically lowers analysis time for this as well as a larger model.

Due to the number of factors that affect a separation in Capillary Electrophoresis, a factorial design presents an interesting choice for a thoroughly systematic method development. Separation voltage, temperature, buffer type, buffer pH, buffer ionic strength, column length, organic modifier concentration, injection type, and injection setpoints are nine relevant factors. To investigate the main and interaction effects of these nine factors with a full factorial design, k = 9, and 2^9 or 512 runs would be needed. It is possible to lower this number, utilizing a fractional factorial design (55, 56).

Fractional Factorial Design.

There are several advantages to utilizing a fractional factorial design when the numbers of experimental variables in a factorial investigation are large. There is little loss of information while significantly lowering the length of experimentation (52, 53). Consider the design of a 2^5 factorial experiment (Table 2). This investigation would

Table 2. 2^5 Factorial design. (Five factors at two levels, centerpoints excluded).

Run	Factor A	Factor B	Factor C	Factor D	Factor E	Response (X)
1	-	-	-	-		X_1
2	+	-	-	-	-	X_2
3	-	+	-	-	-	X_3
4	+	+	-	-	-	X_4
5	-	-	+	-	-	X_5
6	+	-	+	-	-	X_6
7	-	+	+	-	-	X_7
8	+	+	+	-	-	X_8
9	-	-	-	+	-	X_9
10	+	-	-	+	-	X_{10}
11	-	+	-	+	-	X_{11}
12	+	+	-	+	-	X_{12}
13	-	-	+	+	-	X_{13}
14	+	-	+	+	-	X_{14}
15	-	+	+	+	-	X_{15}
16	+	+	+	+	-	X_{16}
17	-	-	-	-	+	X_{17}
18	+	-	-	-	+	X_{18}
19	-	+	-	-	+	X_{19}
20	+	+	-	-	+	X_{20}
21	-	-	+	-	+	X_{21}
22	+	-	+	-	+	X_{22}
23	-	+	+	-	+	X_{23}
24	+	+	+	-	+	X_{24}
25	-	-	-	+	+	X_{25}
26	+	-	-	+	+	X_{26}
27	-	+	-	+	+	X_{27}
28	+	+	-	+	+	X_{28}
29	-	-	+	+	+	X_{29}
30	+	-	+	+	+	X_{30}
31	-	+	+	+	+	X_{31}
32	+	+	+	+	+	X_{32}

require the collection of 32 data points. Generating a fractional factorial design of a ¼ fraction of this table would require only 8 experimental runs (Scheme 2), with a

$$2^{(k-s)} = 2^{(5-2)} = 2^3 = 8$$

Where k = number of factors and s is derived from the equation:

$$1/4 = (1/2)^s$$

Where the fraction = 1/4.

Scheme 2. Generation of a ¼ fractional factorial design of five factors at two levels (k = 5, s = 2).

minimum of information loss due to confounding. Any 3 (k-s) factors are set up in a full factorial experiment. It is first necessary to determine design generators (53). Design generators represent the products of multiplying together a combination of the + and values from these first three columns. It is necessary to utilize s number of unique generators for a fractional factorial design; these generators must not be the products of any other generator combination (Scheme 3). The values for the fourth factor column are produced

$$D = AC, E = AB$$

$$AC * AB = AACB = CB$$

Scheme 3. Design generators for factors D and E. The implicit design generator is CB, the unique product of DE.

by multiplying the + or - in columns A and C, corresponding to the design generator for variable D and resulting in a + or -. The values for the fifth factor column are then

produced by multiplying the + or – in columns A and B, corresponding to design generator E. The result is a ¼ fraction of a 2^5 factorial design (Table 3).

Table 3. ¼ fraction of a 2^5 factorial design. Generators D = AC, E = AB.

Run	Factor A	Factor B	Factor C	Factor D	Factor E	Response (X)
1	-	-	-	+	+	X_1
2	+	-	-	-	-	X_2
3	-	+	-	+	-	X_3
4	+	+	-	-	+	X_4
5	-	-	+	+	-	X_5
6	+	-	+	+	-	X_6
7	-	+	+	-	+	X_7
8	+	+	+	+	+	X_8

There are three additional quarters that can be represented by the design generators D = -AC and E = -AB, D = AC and E = -AB, or D = -AC and E = AB. The quarter chosen to be utilized is arbitrary.

It is important to understand and account for confounding factors. If several measurements are made when two factors are at the lower level, and also when the two factors are at a high level, but not when the factors are at different levels, the effect due to the individual factors can not be estimated. Any information lost in a fractional design can be attributed to these confounding factors.

Resolution of a fractional factorial design is determined as the minimum number of variables employed in the design generators, a number that relates to the confounding of factor interactions. In the example above for a ¼ fraction of a 2^5 design, the resolution (R) is calculated as R = 2. Effects having an order less than 2 are not confounded with any other effect with order less than t = R – e factors (53). In a design with a resolution

of 4, main effects (e = 1) are not confounded with any other main effect (t = 1) or two-factor interaction (t = 2). Two-factor interactions (e = 2) are not confounded with main effects but may be confounded with other two factor interactions (53).

When approaching a complex analytical technique such as capillary electrophoresis, it may be beneficial to utilize a fractional factorial design toward method development, ruling out a large number of influential factors with a limited amount of experiments.

Summary.

At this time, there have been no reported studies concerning the usefulness of capillary electrophoresis in the qualitative and quantitative analysis of yohimbine from either commercially available products or biological fluids. This paper will deal with the investigation of capillary electrophoresis as an analytical tool for the qualitative and quantitative analysis of yohimbine in commercially available products. In addition, the use of a factorial experimental design will be evaluated to investigate nine important factors that influence capillary electrophoresis method development.

DEFINITIONS IN CAPILLARY ELECTROPHORESIS.

Area.

The area under a peak on an output electropherogram.

Capillary Effective Length (l).

Length of the capillary employed from inlet to detector window.

Capillary Total Length (L).

Length of the capillary employed from inlet to outlet.

Electrophoretic Mobility (μ_e).

$$\mu_e = q/(6\pi\eta r)$$

Where: q = ion charge
η = solution viscosity
r = ionic radius

Electrophoretic Velocity (ν_{ep}).

This value is calculated by dividing the effective capillary length by the migration time

Joule Heating.

The resultant heating of a conductive medium upon application of a current flow.

Field Strength (E).

$$\frac{V}{L}$$

Where: V = applied voltage
L = total capillary length

Migration Time.

Migration time is described as the amount of time for a compound to travel from the point of injection to the point of detection in capillary electrophoresis. This value is analogous to a retention time in liquid chromatography (LC). However, separations of compounds in CE are a function of individual compound mobilities under an applied voltage, where LC separations are a function of individual compound interactions with a stationary phase.

Resolution (R_s).

Resolution is described as the separation between two peaks, and represented by the equation:

$$R_s = 0.18\Delta\mu_e\frac{(E\ I)}{\mu_{em}}^{1/2}$$

Symmetry.

Symmetry is a measure of peak shape deviation from a Gaussian curve.

Theoretical Plates (N).

Theoretical plates are the chromatographic determination of separation efficiency.

$$N = \frac{\mu_e EI}{2D}$$

Where: μ_e = Electrophoretic mobility
D = Diffusion coefficient of the solute in the buffer system
E = Electric Field
I = Column effective length

Zeta Potential (ζ).

The zeta potential is a measurement of the potential difference between the capillary wall and the buffer solution within.

this waste removal. Renewed interest surfaced at this time for the development of microbore column chromatography in an effort to reduce solvent waste. The development of this technique theoretically lowers experimental costs while maintaining resolution (50). A combination of the positive aspects of these techniques are employed in capillary electrophoresis, which utilizes a charge based separation in a microbore column.

Hjerten described the first capillary electrophoresis design in 1967 while at the University of Uppsala in Sweden (51). Utilizing 1- to 3-mm internal diameter quartz capillaries coated with methylcellulose, separations of inorganic ions, proteins, nucleic acids, and microorganisms were demonstrated by free zone electrophoresis and later, isoelectric focusing. Capillary technology appeared to be detrimental to the evolution of this technique, as variations in optical pathway and large column diameters were the limiting factors of performance (50). Gel techniques remained superior for these applications due to the availability of the necessary technology at the time.

During the latter part of the 1980's, the investigation of capillary electrophoresis advanced due to the useful advantages of small amount of solvent employed and the qualitative precision obtained in analysis of biopharmaceuticals. This evolution was aided by the technology found in gas chromatography, in the form of small internal diameter fused silica capillaries, and from advances in HPLC, namely improved detector and autosampler capabilities. CE has since emerged as a technique complimentary to HPLC that offers reliable, efficient separations for several unique applications in the biotechnology and biopharmaceutical fields. The separation of structural proteins (49, 50), restriction digests (49, 50), and PCR products (50) are only a few of the examples that this expanding technique envelopes.

Theory of Capillary Electrophoresis.

Capillary electrophoresis (CE) is the family of separation techniques based on the mobility differences of charged species in free solution under an applied voltage. Typically, a fused silica capillary with an internal diameter between 25 and 100 μm is placed between two buffer reservoirs. The capillary is filled with buffer, then a high voltage is applied to the electrodes in each buffer reservoir, creating an electric field across the length of the capillary (Figure 2). It is within this electric field that a separation based on charge to size ratios is afforded. Detection of resolved analytes is achieved on-column through a window on the capillary from which the external coating has been removed.

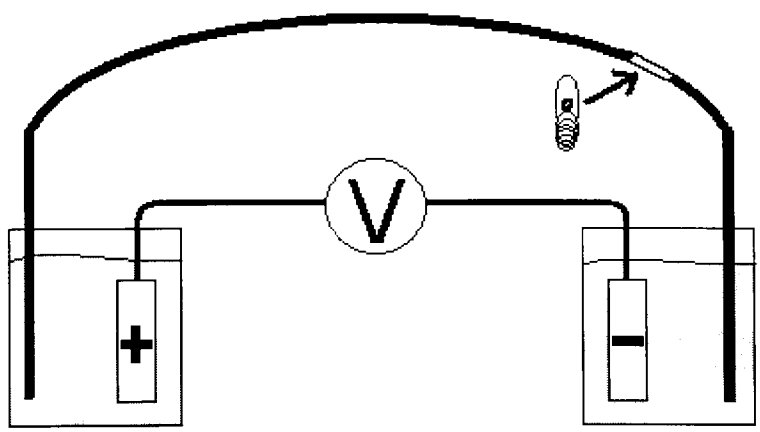


Figure 2. CE Schematic

Electrophoretic Mobility.

Electrophoretic mobility (μ_e) is described as the velocity that an ion demonstrates under an applied voltage. Magnitude of mobility is a function of separation buffer pH effect on analyte charge. Theoretically, anions have μ_e towards the anode, cations have μ_e toward the cathode, and neutral analytes will demonstrate no μ_e. However, in the capillary electrophoretic separation of a mixture of cations, anions, and neutrals, at a neutral or alkaline pH, peaks characteristic for each species will be seen on the resultant electropherogram. This result infers that regardless of charge, each ion will have a net mobility toward the cathode. The phenomenon that contributes to this peculiar effect is called electroosmotic flow (EOF).

Generation of Electroosmotic Flow.

Electroosmotic flow (electroosmosis) (Equation 1) is described as a bulk flow of

$$V_{eo} = \frac{\varepsilon \xi E}{4\pi\eta} \qquad 1$$

Where: ε = dielectric constant of the buffer
η = viscosity of the buffer
ξ = zeta potential measured at the capillary wall

buffer solution under an applied voltage. This flow is directed toward the cathode in a bare fused silica capillary when buffer pH is above three.

As buffer pH is increased between three and eight, the walls of the fused silica capillary are proportionally deprotonated, inferring a negative charge on oxygen at the capillary surface. Above a pH of eight, the silica is assumed to be completely

MATERIALS AND METHODS.

Reagents.

Standards.

Standard	Company	CAS #	EEC #
Yohimbine hydrochloride	Sigma	65-19-0	200-600-4
Reserpine	Sigma	50-55-5	200-047-9
Eserine (Physostigmine) hemisulfate salt	Sigma	64-47-1	200-585-4

Buffer Components.

Buffer Component	Company	CAS #
o-Phosphoric Acid 85%	Fisher Scientific	7664-38-2
Sodium phosphate, monobasic, monohydrate	J.T. Baker	10049-21-5
Sodium phosphate, dibasic, anhydrous	J.T. Baker	7758-11-4
Citric acid, anhydrous	J.T. Baker	77-92-9
Sodium dihydrogen citrate anhydrous	Fluka Chemica	18996-35-5
Sodium hydrogencitrate sesquihydrate	Aldrich Chemical	6132-05-4
Methanol	J.T. Baker	67-56-1

Capillary Electrophoresis Reagents.

CE Reagent	Company	Part #
1.0 N Sodium Hydroxide Solution for HPCE	Hewlett Packard	5062-8576
0.1 N Sodium Hydroxide Solution for HPCE	Hewlett Packard	5062-8575
Water for HPCE	Hewlett Packard	5062-8578

Instrumentation.

Capillary Electrophoresis

Hewlett Packard HP 3D-CE, Model # G1602A, Serial # 3546G00733

Computer:

Hewlett Packard Vectra XM series 3 5/90.

Control Program:

Hewlett Packard HP 3D-CE Chemstation. Revision A.05.04 [273]

Labline Environmental Orbital Shaker.

Rotary Evaporator:

Labconco Centrivap Concentrator. Catalog # 78100-00

Labconco Cold Trap. Catalog # 78110-00

Fisher Scientific Vacuum Pump. Maxima C Plus. Model M4C.

pH Meter:

VWR Scientific. Model 8005

Beckman Combination Electrode 39846. Lot 59098

Experimental Supplies.

Capillary:

Polymicro Technologies. 25μm Internal Diameter Fused Silica capillary, 360 μm Outer Diameter Polyimide coating. Part #: 2000011

Syringes:

Becton Dickinson and Company: 10 cc syringe, 2.5 cc syringe

Filter discs:

Acrodisc, sterile syringe filter. Pore size: 0.2μm, Diameter: 25mm. HT Tuffryn membrane. Product # 4142

Commercially Available Products:.

Action Labs, Farmingdale, NY.

Yohimbe Powermax 2000- 50 capsules
Pure Yohimbe Bark Extract. Maximum Potency. Fast Acting.
Yohimbine Bark Extract Complex: 2000 mg/ 2 Capsules

Yohimbe Powermax 2000- 2 fluid ounces
Pure Yohimbe Bark Extract. Maximum Potency. Fast Acting.
Extract of Yohimbe Bark: 2000 mg/ 2ml

General Nutrition Corporation, Pittsburgh, PA.

Men's Yohimbe 451- 60 Capsules
Standardized Herbal Support Preparation.
Yohimbe Bark Extract (Pausinystalia yohimba): 451 mg/ Capsule
(2% Yohimbine Alkaloid = 9 mg)

Great American Nutrition, Salt Lake City, UT

Men's Performance- 20 Tablets
Herbal Formula. Intimacy Formula for Men. Maximum Potency.
Yohimbe (Pausinystalia yohimbe) (bark): 250 mg/ Tablet

Irwin Naturals, Culver City, CA.

Yohimbe-Plus- 30 Extra Large Tablets
Five High Potency Herbs for Male Drive and Stamina
2000 mg Daily Supply of Yohimbe. Guaranteed Potency.
Yohimbe Bark Powder: 2000 mg/ 3 Tablets

Natrol, Chatsworth, CA.

Yohimbe Bark. Whole Herb Powder- 90 Capsules
Yohimbe, powdered (bark): 500 mg/ Capsule

Only Natural, Island Park, NY.

Yohimbe 1000 plus- 10 tablet trail pack
For Men Only Booster. 100% Pure African Yohimbe Bark.
Yohimbe Bark. (100% Pure African Bark): 1000 mg/ 2 Tablets

Power Force, Hauppauge, NY.

Male Formula. Performance Enhancer- 30 Capsules
100% Pharmaceutical Grade.
Yohimbe Bark Extract: 800 mg/ 3 Capsules

Smart Health USA, Beverly Hills, CA.

V Herbal Ultra- 30 tablets
Ultra Pleasure Delivery System. The All Natural Alternative.
Yohimbe Extract 2%: 250 mg/ 2 Tablets

Saint Rose Heights, Norwalk, CT.

Sobe Energy- 20 fl oz.
Energy drink
Guarana, Yohimbe, Arginine

Twinlab, Ronkonkoma, NY.

Yohimbe Fuel- 50 capsules
Guaranteed Potency Yohimbe Bark Extract.
Standardized for Yohimbine.
Yohimbine: 8 mg/ capsule

Male Fuel- 60 capsules
Male Formula. With Yohimbe Bark Extract.
Standardized for Yohimbine.
Yohimbe Bark Extract: 800 mg/ 6 Capsules

Powerman Yohimbe Power- 100 Capsules
The All Night Long Pill. Pure Yohimbe Bark Extract.
Yohimbe Bark Extract: 225 mg/ Capsule
Standardized for 4.5 mg yohimbine alkaloids

Powerman Yohimbe Stack- 30 Tablets
The All Night Long Pill. Pure Yohimbe Bark Extract.
Yohimbe Bark Extract: 225 mg/ Tablet
Standardized for 4.5 mg yohimbine alkaloids

Factorial and Fractional Factorial Design.

Statistical Design Software.

Minitab 12 for Windows. Release 12.21. Minitab Inc. 1998

Methods.

Experimental Design.

Run voltage, temperature, buffer composition, buffer pH, buffer ionic strength, injection type, injection setpoint, organic modifier concentration, and effective column length comprised the factors chosen for fractional factorial design one (Table 4).

A two-level, one-quarter fractional factorial experimental design at resolution four was compiled using design generators provided by Minitab (Figure 6). Four centerpoints

Table 4. Factor levels chosen for FFD1. Low level represented by -, high level represented by +, centerpoints represented by 0. Factors D and F based on factors C and E, respectively (i.e. when C is -, corresponding to citrate buffer, D + is 6. When C is +, D + corresponds to phosphate buffer at pH 8).

Factor —>	A	B	C	D(C-)	D(C+)	E	F(E-)	F(E+)	G	H	J
Level	T	kV	Buffer	Citrate pH	Phosphate pH	Injection	Electro-kinetic Inj(kV)	Pressure Inj(mbar)	Column Length	Methanol (%)	Ionic Strength
	25	10	Citrate	3	3	Electro-kinetic	5	20	25	0	0.01
+	35	20	Phosphate	6	8	Pressure	15	50	75	10	0.1
0	30	15	X	4.5	6.5	X	10	35	50	1	0.05

G = ABCD H = ACEF J = CDEF

Figure 6. Design generators for factorial design one.
Generated by Minitab Statistical Software.

were added to complete the design, resulting in 68 experimental runs performed in duplicate (Table 5).

Table 5. Design Matrix for the factorial design one. Design generators G=ABCD, H=ACEF, and J=CDEF. The level of factor G for any run is determined by the levels of factors A, B, C, and D for that run. (example: run three. A=-, B=+, C=-, D=-, E=-, F=- Therefore the level of factor G = ABCD = (-)(+)(-)(-) = -, level of factor H = ACEF = (-)(-)(-)(-) = +)

Run #	A	B	C	D	E	F	G	H	J
1	-	-	-	-	-	-	+	+	+
2	+	-	-	-	-	-	-	-	+
3	-	+	-	-	-	-	-	+	+
4	+	+	-	-	-	-	+	-	+
5	-	-	+	-	-	-	-	-	-
6	+	-	+	-	-	-	+	+	-
7	-	+	+	-	-	-	+	-	-
8	+	+	+	-	-	-	-	+	-
9	-	-	-	+	-	-	-	+	-
10	+	-	-	+	-	-	+	-	-
11	-	+	-	+	-	-	+	+	-
12	+	+	-	+	-	-	-	-	-
13	-	-	+	+	-	-	+	-	+
14	+	-	+	+	-	-	-	+	+
15	-	+	+	+	-	-	-	-	+
16	+	+	+	+	-	-	+	+	+
17	-	-	-	-	+	-	+	-	-
18	+	-	-	-	+	-	-	+	-
19	-	+	-	-	+	-	-	-	-
20	+	+	-	-	+	-	+	+	-
21	-	-	+	-	+	-	-	+	+
22	+	-	+	-	+	-	+	-	+
23	-	+	+	-	+	-	+	+	+
24	+	+	+	-	+	-	-	-	+
25	-	-	-	+	+	-	-	-	+
26	+	-	-	+	+	-	+	+	+
27	-	+	-	+	+	-	+	-	+
28	+	+	-	+	+	-	-	+	+
29	-	-	+	+	+	-	+	+	-
30	+	-	+	+	+	-	-	-	-
31	-	+	+	+	+	-	-	+	-
32	+	+	+	+	+	-	+	-	-
33	-	-	-	-	-	+	+	-	-
34	+	-	-	-	-	+	-	+	-
35	-	+	-	-	-	+	-	-	-
36	+	+	-	-	-	+	+	+	-
37	-	-	+	-	-	+	-	+	+
38	+	-	+	-	-	+	+	-	+
39	-	+	+	-	-	+	+	+	+
40	+	+	+	-	-	+	-	-	+

41	-	-	-	+	-	+	-	-	+
42	+	-	-	+	-	+	+	+	+
43	-	+	-	+	-	+	+	-	+
44	+	+	-	+	-	+	-	+	+
45	-	-	+	+	-	+	+	+	-
46	+	-	+	+	-	+	-	-	-
47	-	+	+	+	-	+	-	+	-
48	+	+	+	+	-	+	+	-	-
49	-	-	-	-	+	+	+	+	+
50	+	-	-	-	+	+	-	-	+
51	-	+	-	-	+	+	-	+	+
52	+	+	-	-	+	+	+	-	+
53	-	-	+	-	+	+	-	-	-
54	+	-	+	-	+	+	+	+	-
55	-	+	+	-	+	+	+	-	-
56	+	+	+	-	+	+	-	+	-
57	-	-	-	+	+	+	-	+	-
58	+	-	-	+	+	+	+	-	-
59	-	+	-	+	+	+	+	+	-
60	+	+	-	+	+	+	-	-	-
61	-	-	+	+	+	+	+	-	+
62	+	-	+	+	+	+	-	+	+
63	-	+	+	+	+	+	-	-	+
64	+	+	+	+	+	+	+	+	+
65	0	0	0	0	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0
67	0	0	0	0	0	0	0	0	0
68	0	0	0	0	0	0	0	0	0

The secondary investigation focused on three factors at two levels, buffer pH, buffer ionic strength, and run voltage (Table 6).

A full factorial design of 2^3 experimental runs and an additional two centerpoint runs were completed, totaling ten runs in duplicate (Table 7).

Table 6. Factor levels chosen for factorial design two. (Low level represented by -, high level represented by +, centerpoints represented by 0)

Factor -->	A	B	C
Level	pH	Voltage	Ionic Strength
	4	10	0.01
+	5	20	0.1
0	4.5	15	0.05

Table 7. Design matrix for factorial design two.

Run #	Level A	Level B	Level C
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+
9	0	0	0
10	0	0	0

Preparation of Buffers for Capillary Electrophoresis.

Citrate and Phosphate buffers employed in this investigation were prepared utilizing the Henderson-Hasselbach (Equation 4) and ionic strength (Equation 5) equations toward a known pH and ionic strength (Scheme 4).

$$pH = pKa + \log \frac{[\text{conjugate base}]}{[\text{conjugate acid}]} \quad (4)$$

$$I = 0.5\Sigma (c_i z_i^2) \quad (5)$$

Buffers that did not measure to the calculated pH were recalculated with a correction factor equal to that of the deviation (i.e. a buffer with a measured pH of 5.2 has a deviation of 0.2 pH units. 0.2 is the correction factor. The buffer is recalculated towards a pH of 4.8.)

All buffer solutions were filtered through a 0.2 μm Acrodisc syringe

Citrate buffer: pH = 5.00, I = 0.10, pKa_2 citrate = 4.76

$[H_2A^-] = C_6H_7O_7^-$ $[HA^{=}] = C_6H_6O_7^+$

$0.10 = 0.5\ (([H_2A^-](1^2) + ([Na_1^+](1^2)) + ([HA^{=}](2^2) + 2[Na_2^+](1^2)))$

Setting $[H_2A^-] = [Na_1^+] = x$ and $[HA^{=}] = [Na_2^+] = y$

$0.10 = 0.5\ (((x) + (x)) + ((4y) + (2y)))$

Solving for x

$x = 0.10 - 3y$ 6

The Henderson-Hasselbach equation for these conditions is

$5.00 = 4.76 + \log (y/x)$

Solving for y/x yields

$10^{0.24} = (y/x)$

Substituting Equation 6 for x

$1.738 = y/(0.10 - 3y)$

Solving for y yields

$y = 0.028\ M = [HA^{=}]$

Substituting y into Equation 6

$x = 0.016\ M = [H_2A^-]$

Scheme 4: Buffer calculations for citrate buffer pH 5.00, I = 0.10.

Preparation of Standards for Capillary Electrophoresis.

Yohimbine and Eserine Standard (Factorial Design One).

A measured amount of yohimbine was quantitatively transferred to a 10 mL volumetric flask. Buffer corresponding to the proper pH and ionic strength for the experimental run was added to volume and mixed to yield a 2000 ppm standard.

A measured amount of eserine was quantitatively transferred to a 10 ml volumetric flask. Buffer corresponding to the proper pH and ionic strength for the experimental run was added to volume and mixed to yield a 2000 ppm standard.

Equal parts 2000 ppm yohimbine standard and 2000 ppm eserine standard were added to a 10 ml conical plastic graduated centrifuge tube and vortexed to yield a standard of 1000 ppm in both yohimbine and eserine. The standard solution was then filtered through a 0.2 μm Acrodisc syringe filter disc.

Yohimbine and Eserine Standard (Factorial Design Two).

Deionized ultra filtrated water was employed to utilize the stacking effects that occur when a buffer of lower ionic strength than the separation buffer is present in the injection vial (50). A measured amount of yohimbine was quantitatively transferred to a 10 ml volumetric flask. Deionized water was added to volume and mixed to yield a 2000 ppm standard.

A measured amount of eserine was quantitatively transferred to a 10 ml volumetric flask. Deionized water was added to volume and mixed to yield a 2000 ppm standard.

Equal parts 2000 ppm yohimbine standard and 2000 ppm eserine standard were added to a 10 mL conical plastic graduated centrifuge tube and vortexed to yield a standard of 1000 ppm in both yohimbine and eserine. The standard solution was then filtered through a 0.2 μm Acrodisc syringe filter disc.

Methods of Extraction.

Extraction of Yohimbine from Liquid Products.

Portions of 1 ml of ethanol based liquid products were evaporated with a centrivap concentrator. The resulting solids were reconstituted with deionized water and filtered with a 0.2 μm Acrodisc syringe filter. An aliquot of the filtrate was combined with an aliquot of internal standard and buffer and injected onto the column.

Water based products were filtered with a 0.2 mm Acrodisc syringe filter, and an aliquot of the filtrate was combined with an aliquot of internal standard and buffer and injected onto the column.

Extraction of Yohimbine from Solid Products.

Capsules were broken open and the cellulose casing discarded. Portions of the powders removed were utilized for this investigation. Tablets were crushed with a mortar and pestle, and the resultant powder utilized for this investigation.

Extraction Method One.

Portions of 0.1 gram of bark powder (0.1 gram of product) was wet and brought to volume in a 10 ml volumetric flask with deionized water. This slurry was agitated for 20 minutes at room temperature. The contents were then filtered through a 0.2μm Acrodisc syringe filter, and an aliquot of the filtrate was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Two.

Portions of 0.05 gram of bark powder (0.5 gram of product) was wet and brought to volume in a 10 ml volumetric flask with deionized water. This slurry was agitated for 20 minutes at room temperature. The contents were then filtered through a 0.2 mm Acrodisc syringe filter, and an aliquot of the filtrate was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Three.

Portions of 0.1 gram of bark powder (0.1 gram of product) was wet and brought to volume in a 100 ml volumetric flask with deionized water. This slurry was transferred to a 1 L Erlenmeyer flask and agitated for 60 minutes at 60^0 C in a Labline environmental orbital shaker. The contents were allowed to cool, and the liquid decanted and filtered through a 0.2μm Acrodisc syringe filter. An aliquot of the filtrate was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Four.

Portions of 0.1 gram of bark powder (0.1 gram of product) was wet and brought to volume in a 100 ml volumetric flask with deionized water. This slurry was transferred to a 250 ml Erlenmeyer flask and sonnicated for 15 minutes at room temperature. The contents were allowed to cool to room temperature, and the liquid decanted and filtered through a 0.2μm Acrodisc syringe filter. An aliquot of the filtrate was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Five.

Portions of 0.1 gram of bark powder (0.1 gram of product) was wet and brought to volume in a 10 ml volumetric flask with 10 ml of 95% ethanol. This slurry was agitated for 20 minutes at room temperature. The contents were then filtered through a 0.2 μm Acrodisc syringe filter. The ethanol was evaporated with a rotary evaporator concentrator, and the remaining powder reconstituted into 3 ml of deionized water. The solution was then filtered through a 0.2 μm Acrodisc syringe filter, and an aliquot of the filtrate was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Six.

Portions of 0.1 gram of bark powder (0.1 gram of product) was wet and brought to volume in a 10 ml volumetric flask with chloroform and added to a 125 ml separatory funnel. It was equilibrated with 10 ml of deionized water, and the organic layer collected. The aqueous layer was washed with a portion of 10 ml chloroform. The organic layers were combined, and filtered through a 0.2 μm Acrodisc syringe filter. The

chloroform was removed with a rotary evaporator, and the resultant solids reconstituted into 3 ml of deionized water. The solution was filtered through a 0.2 μm Acrodisc syringe filter, and an aliquot was injected onto the column with an aliquot of an internal standard and buffer.

Extraction Method Seven.

Solid phase extraction was performed on an Alltech reverse phase 900 mg C_{18} solid phase extraction cartridge. The cartridge was first conditioned with 3 ml of methanol, followed by 3 ml of deionized water. 1 ml of sample dissolved in deionized water (0.05 g/ 10 ml) was applied. A 5 ml portion of water was pushed through the column, and collected as a fraction. 3 ml fractions of 10% methanol in water, 50% methanol in water, 75% methanol in water, and 100% methanol were collected successively. The methanol was removed from these fractions with a centrivap concentrator. The resultant solids were reconstituted with 1 ml deionized water, and an aliquot was injected onto the column with an aliquot of an internal standard and buffer to determine the target fraction.

Extraction Method Eight.

Solid phase extraction was performed on an Alltech reverse phase 300 mg C_{18} solid phase extraction cartridge. The cartridge was first conditioned with 3 ml of methanol, followed by 3 ml of deionized water. 1 ml of sample dissolved in deionized water (0.05 g/ 10 ml) was applied. A 3 ml portion of water was pushed through the column, and collected as a fraction. 1 ml fractions of 10% methanol in water, 50%

methanol in water, 75% methanol in water, and 100% methanol were collected successively. The methanol was removed from these fractions with a centrivap concentrator. The resultant solids were reconstituted with 1 ml deionized water, and an aliquot was injected onto the column with an aliquot of an internal standard and buffer to determine the target fraction.

Extraction Method Nine.

Solid phase extraction was performed on an Alltech reverse phase 900 mg C_{18} solid phase extraction cartridge. The cartridge was first conditioned with 3 ml of isopropanol, followed by 3 ml of deionized water. 1 ml of sample dissolved in deionized water (0.05 g/ 10 ml) was applied. A 5 ml portion of water was pushed through the column, and collected as a fraction. Two successive 3 ml fractions of 100% isopropanol were collected, followed by two successive 3 ml fraction of 2% ammonium hydroxide in isopropanol. The isopropanol was removed from 1 ml portions of these fractions with a centrivap concentrator. The resultant solids were reconstituted with 1 ml deionized water, and an aliquot was injected onto the column with an aliquot of an internal standard and buffer to determine the target fraction.

Extraction Method Ten.

Solid phase extraction was performed on an Alltech reverse phase 300 mg C_{18} solid phase extraction cartridge. The cartridge was first conditioned with 3 ml of isopropanol, followed by 3 ml of deionized water. 1 ml of sample dissolved in deionized water (0.05 g/ 10 ml) was applied. A 3 ml portion of water was pushed through the

column, and collected as a fraction. Two successive 1 ml fractions of 100% isopropanol were collected, followed by two successive 1 ml fraction of 2% ammonium hydroxide in isopropanol. The isopropanol was removed from these fractions with a centrivap concentrator. The resultant solids were reconstituted with 1 ml deionized water, and an aliquot was injected onto the column with an aliquot of an internal standard and buffer to determine the target fraction.

Optimized Extraction Conditions for Commercial Samples.

The protocol of extraction five was expanded for continued investigation. Sample mass and ethanol volume were varied between high and low levels to determine the most efficient extraction conditions. The final conditions for real sample analysis involved the use of 0.05 g sample dissolved into 10 ml of 95% ethanol in a volumetric flask. The ethanol was then decanted and filtered through a 0.2 mm Acrodisc syringe filter. 1 ml aliquots of this slurry were evaporated with a Labconco rotary concentrator (60°C * 45 min). The resultant solids were reconstituted into 1 ml of warm deionized water, filtered through a 0.2 mm Acrodisc syringe filter, and an aliquot injected along with a volume of eserine standard and buffer.

Instrumental.

Conditioning of the Capillary Column.

New capillary columns were preconditioned utilizing a standard method. A 45 minute flush with 1.0 N sodium hydroxide, followed by a 45 minute flush with 0.1 N sodium hydroxide assured a uniform surface on the interior of the capillary walls. This was followed by flushes with CE grade water and then the requisite buffer for the analysis at times of 30 and 45 minutes respectively. An additional 90 minutes of flush time were needed for phosphate buffers to reach equilibrium with the column and reduce background noise during experimental runs.

Column conditioning after each unique set of duplicate runs involved two successive 20 minute buffer flushes. Upon completion of a sequence, columns were flushed with CE grade water to remove buffer salts and maintain capillary composition prior to storage.

Capillary Electrophoresis Methods.

Eighteen unique instrumental methods were employed for the primary factorial design (Table 8). These methods were combined with changes in buffer characteristics (ionic strength, pH, type) and capillary column length to give the necessary conditions in Table 5. Electropherograms were collected at 230 and 280 nm (54).

Three methods were employed for the secondary factorial experimental design (Table 9) in which voltage, buffer pH and buffer ionic strength were the variables. Electropherograms were collected at 215, 220, and 230 nm following the

Table 8. Methods employed for the factorial design one.
Methods 17 and 18 represent centerpoints.

Method #	Method Name	Injection Type	Injection kV or Bar	Injection Time (s)	Run T (°C)	Run V (kV)
1	yoh01	Electrokinetic	5 kV	5	25	20
2	yoh02	Electrokinetic	15 kV	5	25	20
3	yoh03	Electrokinetic	5 kV	5	35	10
4	yoh04	Electrokinetic	15 kV	5	35	10
5	yoh05	Pressure	20 mbar	5	25	20
6	yoh06	Pressure	50 mbar	5	25	20
7	yoh07	Pressure	20 mbar	5	35	10
8	yoh08	Pressure	50 mbar	5	35	10
9	yoh09	Electrokinetic	5 kV	5	25	10
10	yoh10	Electrokinetic	15 kV	5	25	10
11	yoh11	Electrokinetic	5 kV	5	35	20
12	yoh12	Electrokinetic	15 kV	5	35	20
13	yoh13	Pressure	20 mbar	5	25	10
14	yoh14	Pressure	50 mbar	5	25	10
15	yoh15	Pressure	20 mbar	5	35	20
16	yoh16	Pressure	50 mbar	5	35	20
17	yoh17	Electrokinetic	10 kV	5	30	15
18	yoh18	Pressure	35 mbar	5	30	15

analysis of 3D spectra from fractional factorial design one demonstrating maxima for the yohimbine peak near 220 nm.

Table 9. Methods employed for factorial design two.
Method 3 represents a centerpoint.

Method #	Method Name	Injection Type	Injection kV	Injection Time (s)	Run T (°C)	Run V (kV)
1	Yoh10V	Electrokinetic	10	5	30	10
2	Yoh20V	Electrokinetic	10	5	30	20
3	Yoh4_5	Electrokinetic	10	5	30	15

Final investigation of commercial samples and yohimbe bark powder was conducted utilizing a 25 cm effective length capillary column, total length 32.5 cm, with a 20 kV run voltage (Table 10).

Table 10. Method employed for commercial yohimbine sample analysis.

Run Time (min)	Column Effective length (cm)	Injection Type	Injection kV	Injection Time (sec)	Run T (^{O}C)	Run V (kV)
10	25	Electrokinetic	5	5	35	20

RESULTS.

Factorial Design One (Fractional).

A ¼ fractional factorial design investigating nine factors at two levels was accomplished at resolution four. 68 experimental runs (2^{9-2} = 64, and 4 centerpoints = 68) were completed in duplicate according to Table 5. The descriptive statistics of the fractional factorial design are shown in Table 11. A full table of the results

Table 11. Descriptive statistics of response ranges for the factorial design one.

Response	N	Minimum	Maximum	Mean	Median
MigTime(1)	68	0.7	51.2	13.6	8.1
MigTime(2)	68	0.7	59.9	15.7	9.5
Area(1)	68	1.5	150.4	29.6	15.9
Area(2)	68	5.0	344.4	74.2	42.4
Symmetry(1)	68	0.01	22.20	1.22	0.20
Symmetry(2)	68	0.00	9.53	0.48	0.19
Plates(1)	68	120	358699	68852	44556
Plates(2)	68	25	540072	75669	43699
Resolution	68	0.18	28.43	6.70	6.21
Selectivity	68	1.04	1.35	1.13	1.13

are included in the appendix. Using the statistical software Minitab, p values were determined for responses including migration time and peak area of each compound, and the resolution between the peaks characteristic for eserine (peak 1) and yohimbine (peak 2) (Table 12). A value of $p < 0.05$ is considered to be statistically significant.

Temperature.

Temperature has no statistically significant effect in the range chosen for this experimental design.

Table 12. p values of responses from factorial design one. A value of $p < 0.05$ is a significant factor, $p < 0.1$ is relevant (i.e. Mig (1) = Migration time for peak one, corresponding to eserine. Mig (2) = Migration time for peak two, corresponding to yohimbine.)

Factor	Mig(1)	Mig(2)	Area(1)	Area(2)	Resolution
Temperature	0.963	0.659	0.926	0.725	0.079
Voltage	0.000	0.000	0.000	0.000	0.001
Buffer type	0.560	0.295	0.437	0.285	0.929
Buffer pH	0.000	0.000	0.066	0.047	0.000
Injection type	0.515	0.694	0.000	0.000	0.001
Injection setpoint	0.401	0.532	0.000	0.000	0.002
Column eff length	0.000	0.000	0.378	0.774	0.000
Methanol %	0.069	0.048	0.700	0.794	0.172
Ionic strength	0.000	0.000	0.169	0.332	0.000

Voltage.

As the voltage was increased, migration times were lower, the observed peak areas decreased, and resolution increased (Figure 7).

Buffer Type.

Peak symmetry and selectivity are considerably lower when phosphate buffers are employed as opposed to citrate buffers. There is no significant effect of buffer type on migration time or peak area of either compound.

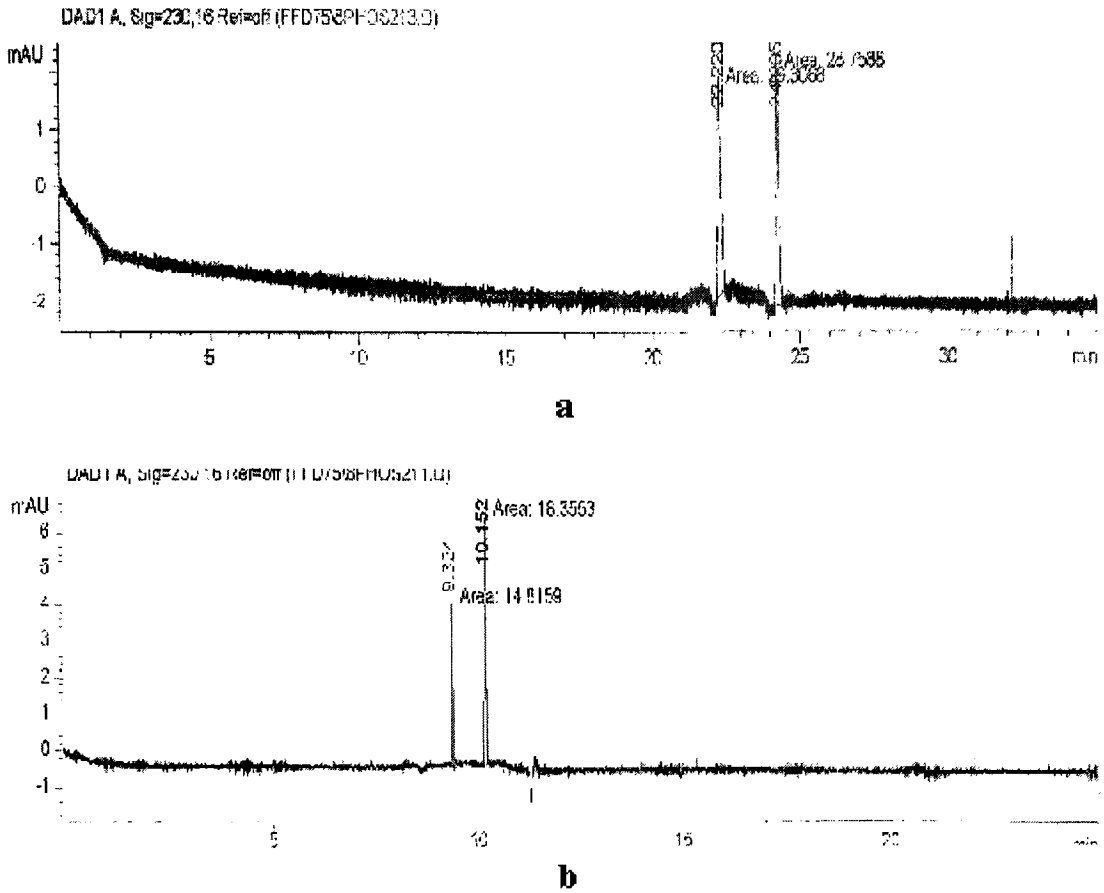


Figure 7. Phosphate buffer pH = 8.0, I = 0.10. Electrokinetic injection (5 kV * 5 sec) Column effective length 75 cm. a) Run 13 Voltage = 10 kV. Area(1) = 24.8, Area(2) = 28.8, R = 10.29; b) Run 16. Voltage = 20 kV. Area(1) = 14.8, Area(2) = 18.4, R = 10.76.

Buffer pH.

Regardless of buffer composition, an increase in buffer pH corresponded to a decrease in migration time and peak symmetry, and an increase in peak area. Resolution decreased at a higher pH (Figure 8).

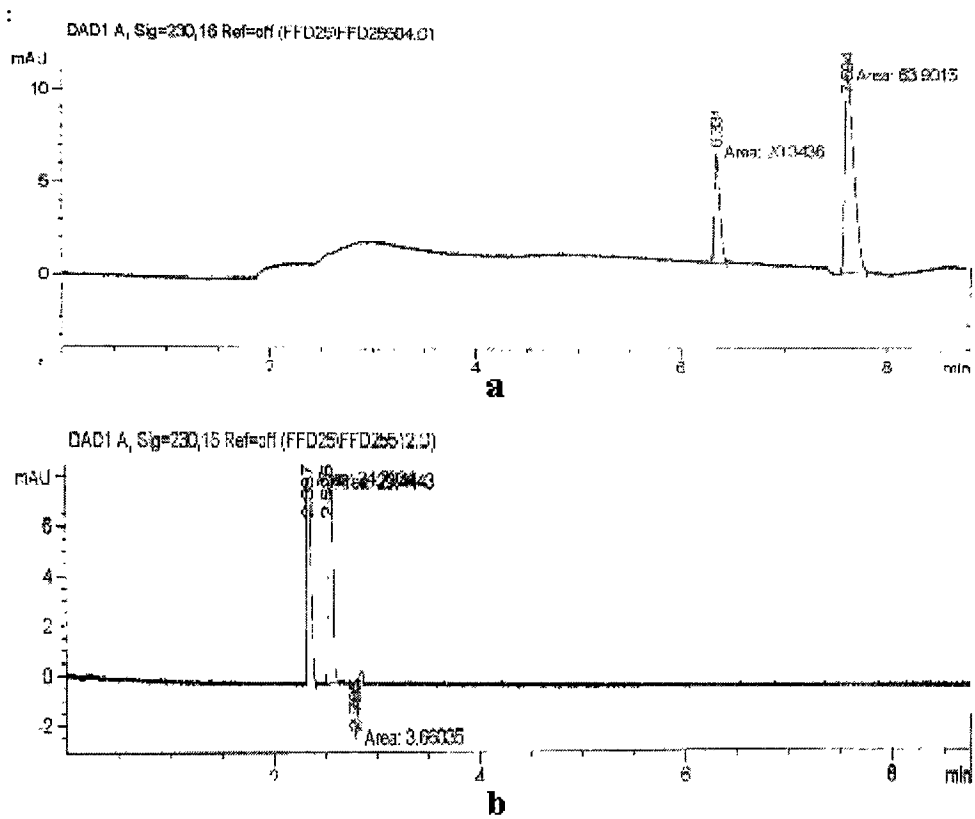


Figure 8. Run voltage = 10 kV, Effective length = 25 cm, Electrokinetic injection (5kV * 5 sec). a) Run 2. Citrate pH 3. Area(1) = 20.3, area(2) = 63.9. R = 10.06. b) Run 14. Phosphate pH 8. Area(1) = 24.8, Area(2) = 29.4. R = 2.01.

Injection Type.

The main effects plot of the peak area results for this design show that the areas of both peak one and peak two were generally lower with the use of pressure injection (Figure 9). Peak areas were higher with the use of electrokinetic injection. Resolution is lower for electrokinetic injection.

Injection Setpoint.

Peak area and peak symmetry increased as a result of an increase in injection level. Resolution decreased as the injection level increased (Figure 9).

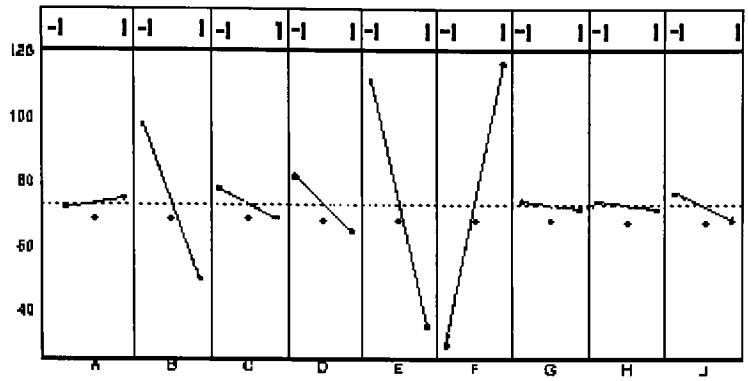


Figure 9. Main effects plot Area(2). This typical output from statistical software denotes the general trend of experimental main effects on the are of peak 2. Trends are read from a lower level (-1) to a higher level (1) of factors A-J in fractional factorial design one. A=temperature, B=voltage, C=buffer type, D=buffer pH, E=injection type, F=injection setpoint, G= column effective length, H=methanol %, I=buffer ionic strength. (E(–1) = electrokinetic injection, (E(1) = pressure injection. F(-1) = low injection level, F(1) = high injection level)

Column Effective Length.

Migration times were higher for the higher-level effective column length of 75 cm (Figure 10). Resolution was also increased by an increase in column length.

Methanol.

The addition of an organic modifier in the form of methanol slightly increased migration times. This did not have a statistically significant effect on resolution between the peaks corresponding to eserine and yohimbine.

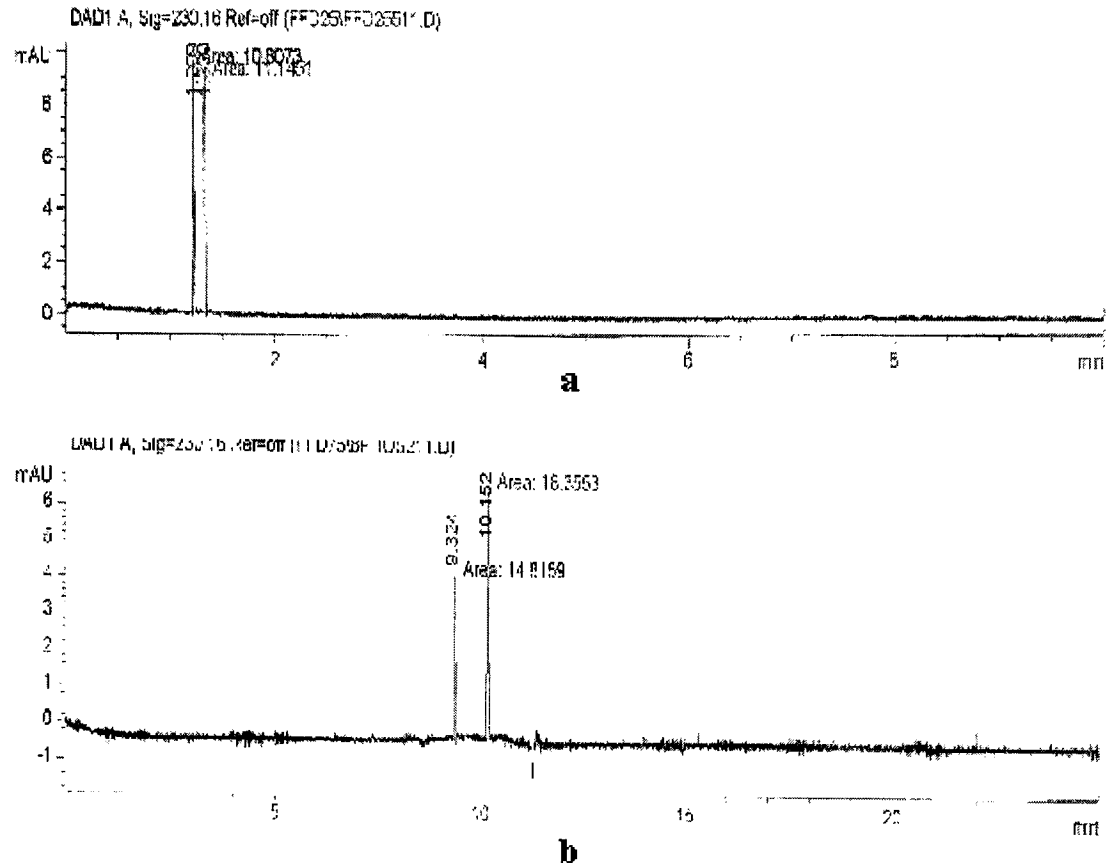


Figure 10. Phosphate buffer pH 8, I = 0.10. Electrokinetic injection (5 kV * 5 sec) a) run 15, 25 cm effective length. R = 4.06. b) Run 16, 75 cm effective length. R = 10.76.

Ionic Strength.

An increase in the ionic strength of the buffer system caused a significant increase in migration time, theoretical plates, selectivity, and resolution (Figure 11). The symmetry of peak one decreased with the increase of ionic strength.

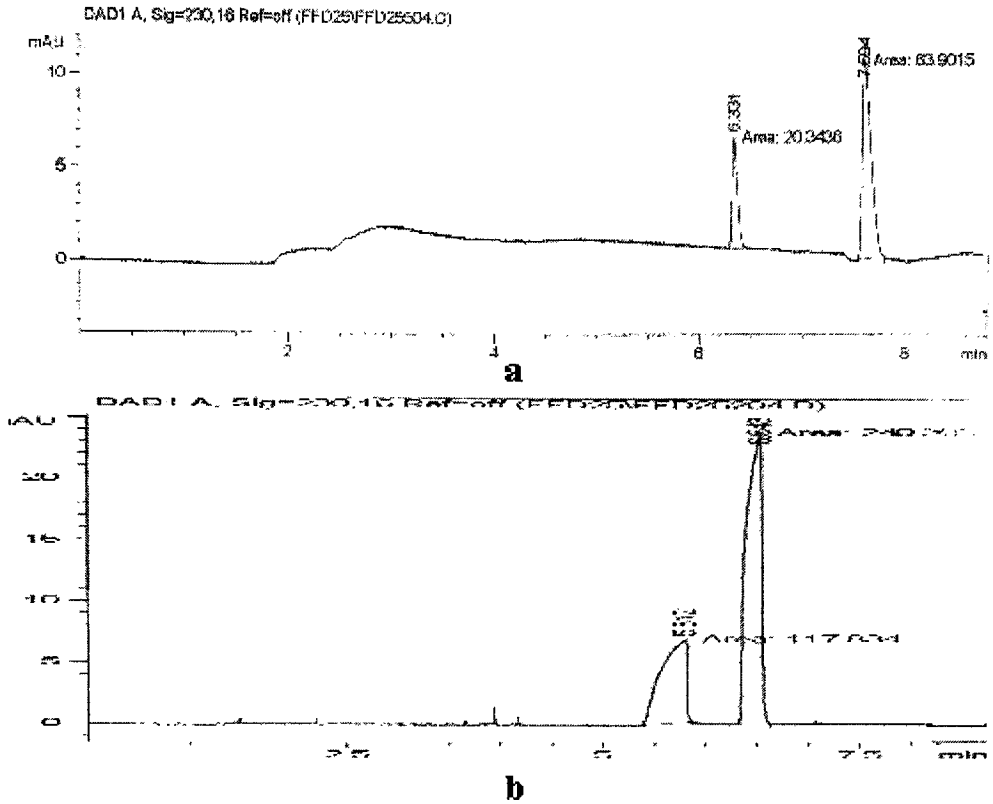


Figure11. Citrate pH = 3. Electrokinetic injection. Run voltage = 10 kV, Effective length = 75 cm. a) Run 2. I = 0.10. Injection = low. b) Run 34. I = 0.01. Injection = high.

Factorial Design Two (Full).

A full factorial experimental design investigating three factors at two levels (2^3 = 8, + 2 centerpoints = 10) was completed. The descriptive statistics of the fractional factorial design are shown in Table 13. A full table of the results are included in the appendix. Using the statistical software Minitab, p values were determined for responses including migration time and peak area of each compound, and the resolution between the peaks corresponding to eserine (peak 1) and yohimbine (peak 2) (Table 14).

Table 13. Descriptive statistics of response ranges for the factorial design two.

Response	N	Minimum	Maximum	Mean	Median
MigTime(1)	10	1.6	6.5	3.5	3.19
MigTime(2)	10	1.8	7.9	4.01	3.62
Area(1)	10	8.2	34.0	16.0	15.1
Area(2)	10	67.7	243.7	122.8	114.5
Symmetry(1)	10	0.05	0.15	0.12	0.13
Symmetry(2)	10	0.03	0.12	0.07	0.06
Plates(1)	10	1146	34762	13079	9572
Plates(2)	10	3905	32197	13856	12530
Resolution	10	1.08	6.61	3.80	3.43
Selectivity	10	1.1	1.22	1.15	1.14

Table 14. p values of responses from factorial design two. A value of $p < 0.05$ is a significant factor, $p < 0.1$ is relevant (i.e. Mig (1) = Migration time for peak one, corresponding to eserine. Mig (2) = Migration time for peak two, corresponding to yohimbine.)

Factor	Mig (1)	Mig (2)	Area (1)	Area (2)	Resolution
Buffer pH	0.260	0.239	0.234	0.012	0.417
Voltage	0.052	0.060	0.096	0.004	0.854
Ionic Strength	0.079	0.082	0.230	0.008	0.010

Voltage.

An increase in voltage corresponded to a decrease of migration time and a decrease in the resultant peak area (Figure 12). There is no significant effect of voltage on resolution in this experimental system ($p = 0.854$). The effect on area ($p_1 = 0.096$) and migration time ($p_1 = 0.052$, $p_2 = 0.060$) are considered relevant, while a significant effect is seen on the area of peak 2 ($p_2 = 0.004$).

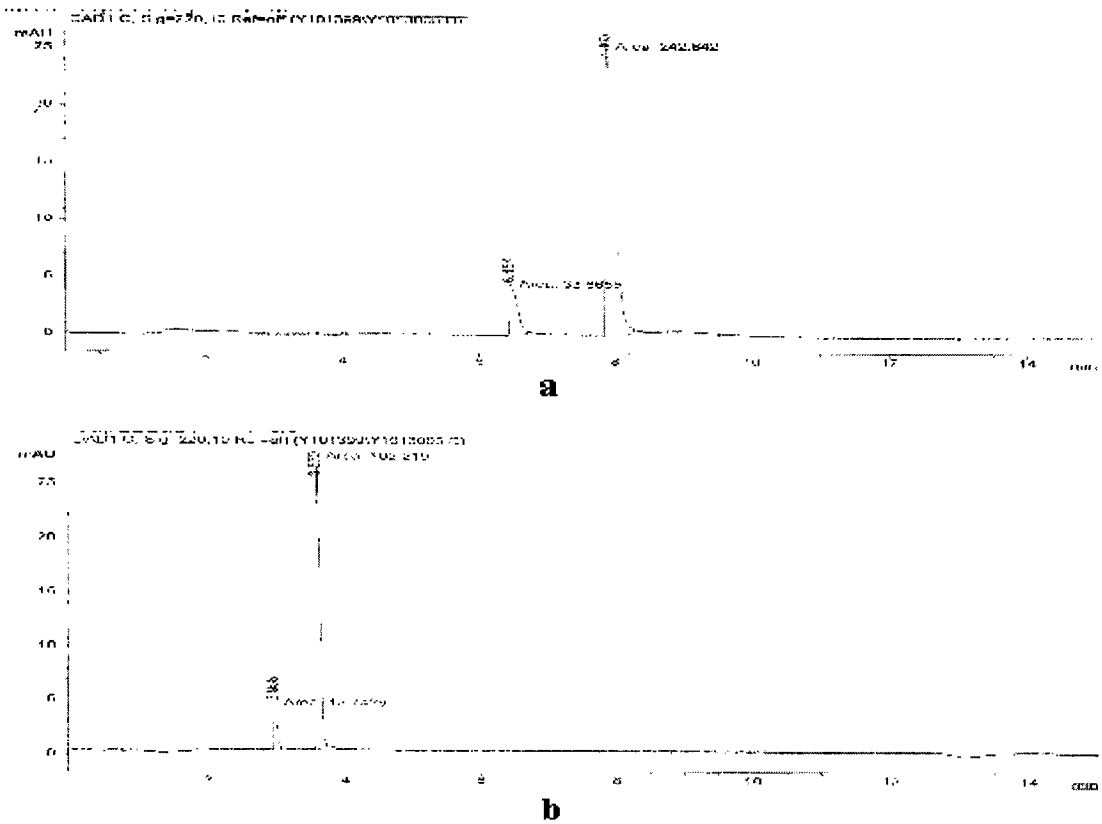


Figure 12. Citrate pH = 4, I = 0.1. a) Run 5, voltage = 10 kV. b) Run 7, voltage = 20 kV.

Buffer pH.

The increase of buffer pH decreased migration time and peak area (Figure 13). There is no mathematically significant effect of pH on resolution in this experimental system ($p = 0.417$). The area of the peak corresponding to yohimbine was significantly effected by a variation of pH in this range ($p_2 = 0.012$). Peak area was demonstrated as higher toward the lower pH.

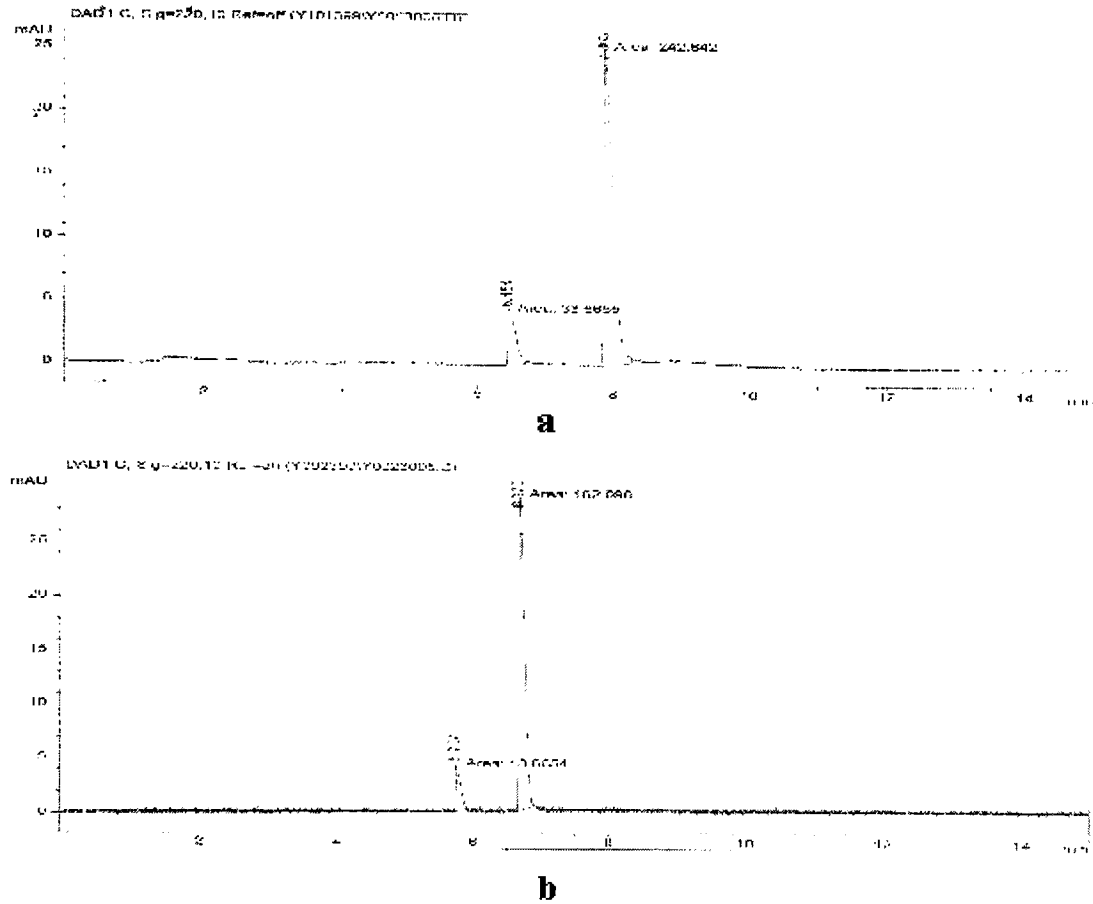


Figure 13. Buffer ionic strength I = 0.1, run voltage = 10 kV. a) pH 4. b) pH 5.

Ionic Strength.

An increase in ionic strength increased migration time, peak area, theoretical plate count, and resolution (Figure 14). The area of the peak corresponding to yohimbine was effected significantly by the variation of ionic strength in this range ($p_2 = 0.008$). Ionic strength had a relevant effect on the migration times of both peaks one and two ($p_1 = 0.079$, $p_2 = 0.082$).

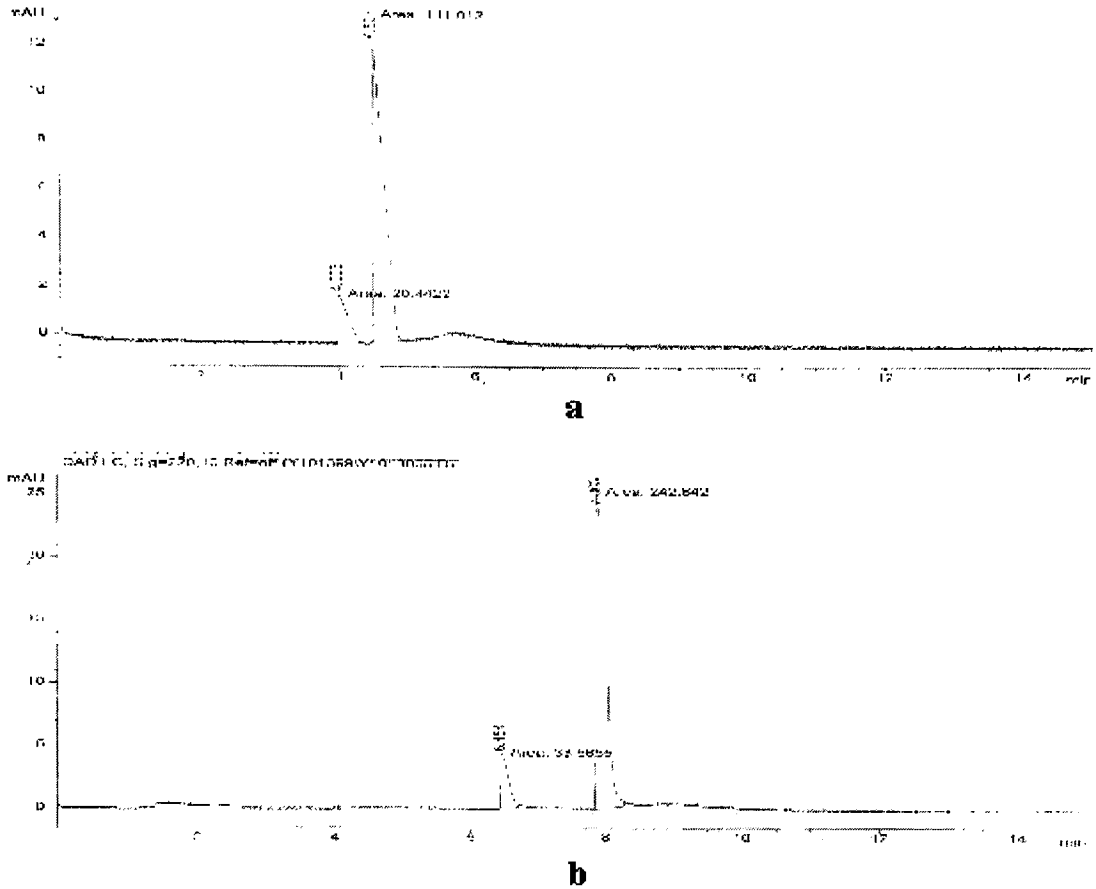


Figure 14. Citrate buffer pH = 4, run voltage = 10 kV. a) Run 1. I = 0.01. b) Run 5. I = 0.1.

Optimized Experimental Conditions.

The separation conditions were chosen as follows: A 25 cm effective length by 25 μm internal diameter fused silica capillary was employed at a temperature of 35° C. The run voltage was set at 20 kV for 10 minutes to afford a resolved separation of yohimbine alkaloids. The buffer chosen was citrate, pH 4.0, diluted 1:10 in sample vials. An electrokinetic injection (5 kV * 5 seconds) was utilized.

Commercially Available Products.

Several products were purchased from local merchants. These products were assigned sample identifications (ID) (Table 15).

Table 15. List of assigned product ID and product potency claim.

Sample ID	Company	Product	Claim
C1	Natrol	Yohimbe Bark: Whole Herb Powder	500 mg yohimbe bark/ capsule
C2	Action Labs	Yohimbe Powermax 2000	1000 mg yohimbe bark extract complex/ capsule
C3	Power Force	Male Formula	266.67 mg yohimbe bark extract/ capsule
C4	GNC	Yohimbe 451	9 mg yohimbine alkaloid/capsule
C5	Twinlab	Yohimbe Fuel	8 mg yohimbine/capsule
C6	Twinlab	Powerman Yohimbe Power	4.5 mg yohimbine alkaloids/ capsule
C7	Twinlab	Male Fuel	133.33 mg yohimbe bark extract/ capsule
T1	Irwin Naturals	Yohimbe Plus	666.67 mg yohimbe bark powder/ tablet
T2	Only Natural	Yohimbe 1000 Plus	500 mg yohimbe bark/ tablet
T3	Twinlab	Powerman Yohimbe Stack	4.5 mg yohimbine alkaloids/ tablet
T4	Smart Health USA	Herbal V Ultra	125 mg yohimbe extract 2%/ tablet
T5	Great American Nutrition	Men's Performance	250 mg yohimbe bark/ tablet
L1	Action Labs	Yohimbe Powermax 2000	1000 mg yohimbe bark extract/ ml
L2	Sobe	Energy	No potency claim on label.
YBP		Yohimbe Bark Powder	up to 6% yohimbine

The method above was employed to determine the amounts of yohimbine in commercially available preparations. Several extraction methods were investigated and compared towards the determination of yohimbine in a number of samples (Table 16).

Extraction five was chosen for further investigation. The amount of product and the amount of solvent were varied from a low to a high level, and a mid point

Table 16. Comparison of ten extraction conditions. The values are reported as a weight percentage of yohimbine in product (Extractions 7-10 were completed for C4 and ybp only.)

Sample ID	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6	Extract 7	Extract 8	Extract 9	Extract 10
C1	0.00	0.00	0.00	0.00	0.00	0.00	*	*	*	*
C2	0.11	0.11	0.11	0.17	0.16	0.06	*	*	*	*
C3	0.47	0.24	0.37	0.34	0.46	0.01	*	*	*	*
C4	0.57	0.60	0.78	1.16	1.60	0.05	0.00	0.00	0.04	0.09
YBP	0.09	0.14	0.16	0.38	0.48	0.02	0.00	0.00	0.03	0.06

was included. The final extraction conditions were chosen at 0.05 g of sample dissolved into 10 ml of ethanol.

The calibration curve for the yohimbine capillary electrophoretic method routinely produced R2 > 0.9989, while allowing quantitation of the yohimbine peak over two orders of magnitude (Figure 15). Calibration points of 0, 1, 5, 10, 25, 50, and 100

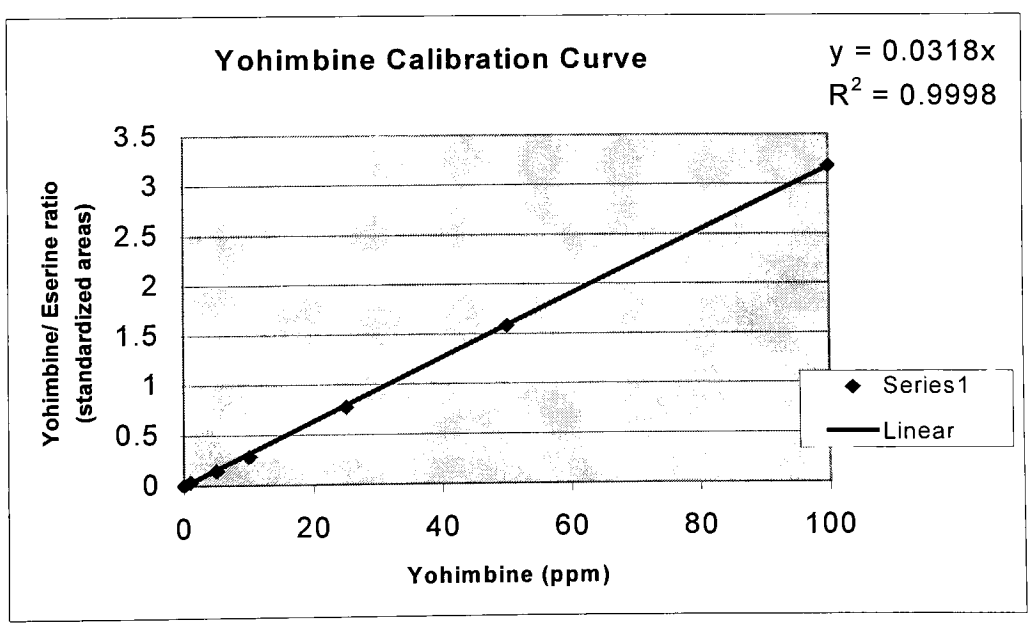


Figure 15. Yohimbine analysis Calibration curve. Samples with detectable yohimbine ranged from 7 ppm yohimbine to 67 ppm yohimbine in the sample vial (Sample vials included buffer and eserine standard)

ppm yohimbine were included. A constant amount of internal standard (eserine) was added to each sample and standard vial, and the time corrected area ratio (Equation 7) between the peaks was utilized for the analysis.

$$\text{yohimbine/eserine ratio} = \frac{(\text{Area of yohimbine peak}/\ \text{Migration time of yohimbine peak})}{(\text{Area of eserine peak}/\ \text{Migration time of eserine peak})} \qquad 7$$

The complete set of samples was extracted under these optimal conditions, and injected onto the column utilizing the determined method (Table 17).

Table 17. Yohimbine in commercially available samples. Values reported as a percent mass of product (excludes cellulose casing of capsules).

Sample ID	Yohimbine (%)	Yohimbine in Product (mg)	Product Claim
C1	0.00	0.00	500 mg bark powder
C2	0.16	1.83	1000 mg extract complex
C3	0.26	1.82	266.67 mg bark extract
C4	1.61	10.67	9 mg yohimbine (2% extract)
C5	1.14	6.28	8 mg yohimbine
C6	0.60	2.94	4.5 mg yohimbine alkaloids
C7	0.19	1.58	133.33 mg bark extract
T1	0.16	2.62	666.67 mg bark powder
T2	0.00	0.00	500 mg bark
T3	0.32	5.12	4.5 mg yohimbine alkaloids
T4	0.06	0.71	125 mg yohimbine extract
T5	0.00	0.00	250 bark
L1	0.04	0.40	1000 mg bark extract
L2	0.00	0.00	No claim
YBP	0.51	*	up to 6 % yohimbine
Aphrodyne	*	**5.40**	**Pharmaceutical Grade**

0.015 g of standard yohimbine was added to an aliquot of sample C1 prior to extraction to determine percent recovery of the extraction method. The extraction was completed, and experimentally determined to have a 99.8 % recovery.

Ten successive injections of sample C7 were completed to evaluate method reproducibility. The peak corresponding to the internal standard (eserine) averaged a standardized area (area/ migration time) of 16.4 mAU/min with a standard deviation of 0.11 mAU/min. The peak corresponding to yohimbine averaged a standardized area of 1.3 mAU/min with a standard deviation of 0.10 mAU/min. When these areas were combined to form the yohimbine/eserine ratio that was employed for data analysis, the average ratio was 0.079. The standard deviation of this number was 0.0009.

DISCUSSION.

Factorial Design One (Fractional).

Nine factors were investigated at two levels in a 1/8 fractional 2^9 factorial design employing four centerpoints. Evaluation of the data was assisted with Minitab Version 12 statistical computing software. A discussion of the relevance of each factor in the range of this examination follows. To review, when $p < 0.05$, an effect was considered statistically significant; when $0.05 < p < 0.1$, the effect was considered relevant.

Temperature.

Temperature was varied between a lower limit of 25° Celsius and an upper limit of 35° Celsius. Temperature does not have a mathematically relevant effect on the experimental outcomes of this system save resolution ($p = 0.079$). A change in system temperature can effect the buffer viscosity and result in modification of the electroosmotic flow. Resolution was experimentally determined to be greater at a higher temperature, an effect that corresponds to a decrease in electroosmotic flow. As the overpowering EOF is lowered, the unique electrophoretic mobilities of each species are increasingly expressed; this effect is demonstrated by an increase in resolution. There were significant two factor interactions involving temperature and buffer type ($p = 0.021$), and temperature and buffer pH ($p = 0.027$) on resolution. Each of these demonstrated a decreased resolution at a high level of both interaction factors. Temperature would have to remain low if the higher level of buffer type and buffer pH were to be employed.

Voltage.

Voltage was varied from 10 kV at the lower level, to an upper level of 20 kV. The maximum voltage that the system can provide is 30 kV. Migration times (p_1 = 0.000, p_2 = 0.000), peak areas (p_1 = 0.000, p_2 = 0.000), and the theoretical plate counts (p_1 = 0.001, p_2 = 0.026) of both compounds, as well as peak resolution (p = 0.001) are significantly effected by the variation of voltage under the constraints of this experimental design. As the voltage increased, migration times decreased. This is consistent with the equation describing electrophoretic mobility. The observed peak areas decreased as the voltage was increased. This phenomenon is due to the increase in electroosmotic flow and electrophoretic mobility. As a compound increases in mobility, it is encountered by the detector for a shorter duration. This results in a seemingly decreased area for the compound under these conditions. It may be a good practice to divide the peak area by migration time to give a time-standardized area. This calculation may limit reproducibility error of peak area due to variation in migration time. As voltage is increased, the theoretical plate count increases as a result of the shorter migration time, since time is a variable in theoretical plate calculations. Resolution increases as voltage is increased in this model system, consistent with the equation describing resolution.

There are several two-factor interactions that involve the separation voltage effect on resolution: buffer type (p = 0.022), buffer pH (p = 0.011), column effective length (p = 0.028), and buffer ionic strength (p = 0.009). A high voltage combined with either a high level buffer type or a high level buffer pH resulted in a decreased resolution. This result

should be expected, as an increase in any of these three individual effects acts to increase the magnitude of the separation buffer electroosmotic flow. A strong EOF can overpower the individual component electrophoretic mobilities, decreasing resolution. A high voltage combined with a high level column effective length or a high level buffer ionic strength worked to increase resolution. In these cases, individual compound mobility characteristics are greater, expressed in either the greater separation distance or the lower conductance, leading to a longer separation time.

The effects of voltage on the resolution of this separation system are complex given the wide range of other experimental variants. Voltage was chosen as a factor in the secondary analysis for this reason.

Buffer Type.

The investigation of buffer composition focused between two commercially available and easily produced buffer types, citrate and phosphate. Peak symmetry (p_1 = 0.000, p_2 = 0.033), theoretical plate count (p_1 = 0.000, p_2 = 0.000), and selectivity (p = 0.002) are significantly effected by the variance of buffer composition. Peak symmetry is considerably lower when phosphate buffers are employed as opposed to citrate buffers. This may be due in part to the complexing reactions that phosphate tends to undergo with the capillary walls (50). The charged walls of the capillary, generators of electroosmotic flow, can be partially or fully masked by this phosphate complex. The loss in peak symmetry may be a function of the loss of uniform capillary walls, resulting in sample-wall interactions. Selectivity was experimentally determined as lower for the phosphate buffers in comparison with the citrate buffers. Generally, citrate is easier to work with

under these experimental conditions. This buffer type has a shorter equilibration time with the column, as well as a lower absorptivity in the wavelength region of interest (200 nm to 230 nm). The only two factor interaction on resolution for buffer type was buffer pH ($p = 0.011$).

Buffer pH.

The pK_a of yohimbine has been experimentally determined as 8.30 (54). The pH of the buffer solution was chosen to range from a lower limit of three to a higher limit of eight based on commercially available buffers. Migration time ($p_1 = 0.000$, $p_2 = 0.000$), peak area ($p_1 = 0.066$, $p_2 = 0.047$), peak symmetry ($p_1 = 0.000$, $p_2 = 0.044$), resolution ($p = 0.000$), and selectivity ($p = 0.000$) were significantly effected by the variation of buffer pH through this range. The area ($p_2 = 0.047$) and theoretical plate count ($p_2 = 0.012$) of the peak corresponding to yohimbine were also effected. Buffer pH has the largest amount of significant effects of all factors included in the first set of experiments.

Regardless of buffer composition, an increase in buffer pH corresponded to a decrease in migration time. This is consistent with the trend of electroosmotic flow to increase as pH rises above three when utilizing a fused silica column. At a low pH ($pH<3$), electroosmotic flow is considered to be low or negligible. An increase in buffer pH corresponded to a decrease in peak areas, correlated to the amount of time required for the compounds to move past the detector. A decline in symmetry is noted at the higher pH levels, which may also be associated with the buffer composition: it was experimentally determined that high pH phosphate buffers have a much lower symmetry then low pH citrate buffers. It is worthwhile to note that runs utilizing high pH citrate

buffer had a lower symmetry than those employing low pH citrate buffer, with the same trend observed for phosphate buffers.

Resolution was decreased at a higher pH of buffer, independent of buffer composition. The electroosmotic flow may overpower the electrophoretic mobilities of the two compounds at the higher pH. At a low pH, where the EOF is not as strong, there is a higher resolution, as the compounds are separated more on the basis of their own characteristic mobilities. There were no additional significant two-factor interactions on resolution involving buffer pH other than those previously discussed.

The effects of pH on the resolution and peak area of this separation system are important considerations in the choice for further work. As pH is increased, resolution, peak area, and migration times decrease. A combination of these results would yield a fast, yet possibly incomplete separation ($R < 1.5$) with lower than maximum peak areas. A separation at a low pH would give an increase resolution, peak area, and a slower migration time. Buffer pH is chosen as a factor in the secondary analysis to find a point between the extremes where each outcome would be optimized: a fast, complete separation with maximum peak areas.

Injection Type.

For the purpose of factorial design one, electrokinetic injection was utilized as the lower level and a pressure injection comprised the upper level of the injection type factor. Each injection type was utilized for two of the four centerpoints. Peak areas ($p_1 = 0.000$, $p_2 = 0.000$), theoretical plate counts ($p_1 = 0.000$, $p_2 = 0.001$), and resolution ($p = 0.001$) are effected by the choice of injection type. Also, the symmetry of peak one was effected

($p_1 = 0.017$). The main effects plot of the peak area results for this design show that the areas of both peak one and peak two were generally lower with the use of pressure injection (Figure 9). Peak areas were higher with the use of electrokinetic injection. This fact demonstrates that the separating species have a high electrophoretic mobility. The movement of these species onto the column is higher under an applied voltage than with the application of a pressure injection. Symmetry is higher for electrokinetic injection. Theoretical plate count is lower for the electrokinetic injection compared to the pressure injection mode. This is consistent with the increased peak area encountered with the electrokinetic injection. Resolution is lower for electrokinetic injection, again consistent with increased peak area. Band broadening in a larger injection plug is consistent with the data encountered in this case. The sample plug in a pressure injection tends to have less dispersion, as all species will have the same velocity as they are mobilized onto the column. A pressure injection is less useful for analytical analysis, as the amount of sample entering the column becomes a function of sample viscosity. There were no significant two-factor interactions on resolution involving injection type. Work needed to be done to increase peak symmetry to increase plate count and reproducibility, leading towards the further investigation of electrokinetic injection for this analysis.

Injection Setpoint.

Under the condition of an electrokinetic injection (E-, Table 4), the lower level was chosen to be 5 kV * 5 seconds. The upper level was set at 15 kV * 5 seconds. When a pressure injection was employed (E+, Table 4), the lower level was set at 20 mbar * 5 seconds, and the upper level was set at 50 mbar * 5 seconds. Peak areas ($p_1 = 0.000$, $p_2 =$

0.000), theoretical plate counts ($p_1 = 0.000$, $p_2 = 0.013$), and resolution ($p = 0.002$) were effected by the variation in injection level. Peak area increased as the injection amount increased. The model does not allow for a determination if this observation was linear, or simply a trend. Peak symmetry increased as a result of an increase in injection level. Theoretical plate count decreased as the injection level increased. Resolution decreased as the injection level increased. An increase in peak area is a result of more analyte introduced on column, consistent with an increase in peak width, which leads to a decrease in resolution. There were no significant two-factor interactions on resolution involving injection setpoint. The setpoint was chosen at a midpoint (electrokinetic injection, 10 kV * 5 sec) for the second analysis under the assumption that the optimized method would include an injection setpoint that offered the greatest peak area while maintaining resolution and peak symmetry. The setpoint meeting these criteria should enable this method to investigate dilute samples.

Column Effective Length.

Column effective length was varied from 25 cm at the low level to 75 cm at the upper level. This variance had a significant effect on migration time ($p_1 = 0.000$, $p_2 = 0.000$), theoretical plate count ($p_1 = 0.000$, $p_2 = 0.000$), and resolution ($p = 0.000$). Migration times were longer for the higher-level column length. Theoretical plate counts were also higher for the longer columns, as this calculation is based in part on column length. Resolution was positively influenced by the increase in column length. Differences in analyte mobility are expressed as an increased resolution as a result of an increase in species migration distance before detection. There was a significant two-

factor interaction on resolution involving column effective length and buffer ionic strength. At a high level of each factor, resolution was increased. This result is consistent with both the effect of a longer capillary column as well as the increase of ionic strength. This is a significant factor due to the effect on the implied separation current. This current is decreased at a higher ionic strength, as well as at a higher column effective length, and the combination of the two factors yields a significant difference. Assuming a resolved separation, the column effective length can be chosen at a lower level to limit migration times. This should assure a short analysis time.

Methanol.

A significant effect on migration time was seen for the peak corresponding to yohimbine ($p_2 = 0.046$) The addition of an organic modifier in the form of methanol tended to decrease migration times. Organic modifiers have been shown to somewhat mask the charge on molecules, either decreasing species mobility, or reducing wall interactions (50). In this case, the wall charges were most likely masked, lowering sample-wall interactions, and decreasing sample migration time. However, the addition of methanol showed no significant effect on either peak area ($p_1 = 0.700$, $p_2 = 0.794$), peak symmetry ($p_1 = 0.284$, $p_2 = 0.496$), or resolution ($p = 0.172$), and was not involved in any significant two factor interactions on resolution. No further investigation of this factor was completed.

Ionic Strength.

Ionic strength was varied over an order of magnitude. Migration times (p_1 = 0.000, p_2 = 0.000), theoretical plate counts (p_1 = 0.000, p_2 = 0.011), resolution (p = 0.000), and selectivity (p= 0.000) of both peaks were effected by this variation of ionic strength. Also, the symmetry of peak one was effected (p_1 = 0.000). An increase in the ionic strength of the buffer system caused a significant increase in migration time, as the EOF is decreased at a higher concentration. Theoretical plate count increased as a result of increased ionic strength. Increased ionic strength causes an increase in the electric field strength, a factor used in the calculation of theoretical plate values. Resolution and selectivity increased with the increased ionic strength, a result of increased migration time. The symmetry of peak one decreased with the increase of ionic strength. This may be due to an increase in band broadening that occurred during the higher migration times. Although there was no significant effect on peak area, and a detrimental effect on peak symmetry, a higher resolution was demonstrated for a higher ionic strength, prompting the further development of this factor. There were no additional significant two-factor interactions on resolution involving ionic strength other than those previously discussed.

Intent of Factorial Design Two.

Conditions were investigated that would allow for maximum peak area and minimum migration time while maintaining a useful resolution. Resolution was determined to be useful above a value of 5, allowing complete separation of the internal standard and sample peaks while assuring an overall robust method. These considerations were satisfied (R_{ave} = 6.3) while the migration times of the two peaks of

interest remained within 0.5 minutes, a duration short enough to assume identical separation conditions across the time frame.

Three factors from the primary investigation stood out as important to these results. Ionic strength, buffer pH, and separation voltage were chosen as the main factors toward the secondary factorial evaluation. The optimum conditions of the six other factors were chosen based on results from the primary investigation.

Temperature had very little impact on the primary investigation. This factor was set at 30° Celsius for factorial design two, slightly above ambient temperature for the room to assure reproducibility. This factor was further adjusted at a later point.

Several responses favored the use of citrate buffers, including theoretical plate count. The preconditioning of columns was much slower when employing phosphate buffers, possibly owing to phosphate buffer salt complexation with the capillary wall. For this reason, citrate buffer was chosen for continued evaluation.

Pressure injection is not very reproducible with the current hardware, and differences in sample viscosity may lead to incorrect measurements. Peak areas from electrokinetic injections were significantly higher than pressure injections at both levels, but there was a resolution loss at the higher level. Electrokinetic injection at a low level (5 kV * 5 sec) proved to be the best injection conditions evaluated in the primary investigation, but may not be feasible when analyzing a very dilute sample. For this reason, the setpoint for injection was chosen at the midpoint (10kV * 5 sec) for the second factorial design, which could be further improved upon at a later time in the investigation.

The addition of methanol as an organic modifier was shown to have no effect on resolution or peak area, and was excluded from further investigation. Methanol may be reintroduced into the experimental conditions in an effort to decrease migration times of the yohimbine peak, assuming resolution is sustained.

The column effective length was determined to be mathematically significant on the outcome of several responses, namely migration time and resolution. There were no significant two-level interactions for this factor, necessitating no further examination in the secondary investigation. Column effective length was chosen to be utilized at the lower level (25 cm) in an effort to limit migration time for the secondary factorial design.

Factorial Design Two (Full).

A 2^3 full factorial design was completed, investigating three factors (separation voltage, buffer pH, and buffer ionic strength) at two levels with two centerpoints. A discussion of the relevance of each factor in the scope of this exploration follows.

Voltage.

Voltage was varied from a lower level of 10 kV to an upper level of 20 kV. No significant main effect or interaction involving voltage was demonstrated in this design other than the effect of voltage on the area of the peak corresponding to yohimbine (p_2 = 0.004). The factor was determined to be relevant to migration time (p_1 = 0.052, p_2 = 0.060) and peak area of the peak corresponding to eserine (p_1 = 0.096). The higher level of separation voltage was consistent with a decrease in migration time and peak area, as the buffer electroosmotic flow and the mobility of each individual compound would be

increased. Resolution was not significantly effected mathematically by the variation of separation voltage in this range ($p = 0.854$). There were no significant two or three factor interactions on resolution involving voltage for this design. The absence of a significant effect of voltage on resolution allows the employment of a high voltage, enabling the shortest possible analysis time. Voltages above this range can be investigated.

Buffer pH.

The pH of citrate buffer was varied from a low level of 4.0 to an upper level of 5.0. An increase in buffer pH significantly decreased the area of the peak corresponding to yohimbine ($p_2 = 0.012$). There was no significant effect on migration time ($p_1 = 0.260$, $p_2 = 0.239$) or resolution ($p = 0.417$). There were no significant two or three factor interactions on resolution involving buffer pH. Further investigation should be orientated toward the lower pH range to maximize yohimbine peak area.

Ionic Strength.

The ionic strength of the citrate buffers employed were varied from a low level of $I = 0.01$, to a high level of 0.1, covering an order of magnitude and the range of commercially available citrate buffers. This factor had a significant effect on both resolution ($p = 0.010$), and the area of the peak corresponding to yohimbine ($p_2 = 0.008$), increasing both. Ionic strength was considered relevant for the lengthening effect on migration times ($p_1 = 0.079$, $p_2 = 0.082$). There were no significant two or three factor interactions on resolution involving ionic strength in this design. An increase in ionic

strength decreases the electroosmotic flow of the buffer as the conductivity of solution is decreased at a high level.

Optimized Experimental Conditions.

Buffer was chosen at pH = 4 to maximize the observed peak area, thereby maximizing method sensitivity to yohimbine. Buffer ionic strength was chosen at I = 0.1 to maximize both resolution and peak area.

Initial sample runs were completed at a run voltage of 20 kV on a 25 cm effective length fused silica capillary. There were four co-migrating peaks witnessed at the time corresponding to the peak for yohimbine. An attempt to better resolve these peaks involved the increase of column effective length to the higher limit investigated, 75 cm, followed by an increase in run voltage to 30 kV. The peaks, although better separated, were still not completely resolved. The increase in effective length had a significant effect on analysis time, jumping to 20+ minutes from just over 3 minutes. The increase in analysis time outweighed the increase in resolution (R_{ave} = 1.25 for a 25 cm column at 20 kV: R_{ave} = 1.35 for a 75 cm column at 30 kV).

Several extraction conditions were investigated (Table 16), and the employed method effectively reduced the concentration of the on-column sample, resulting in a resolved separation. This resolution was maintained at an effective length of 25 cm and run voltage of 20 kV.

Commercially Available Samples.

Samples were randomly chosen from national distributors and purchased from local merchants as listed in the Materials and Methods section. Several extraction conditions were tested on a subsample of four products as well as a portion of yohimbine bark (Table 16). The most complete extraction was afforded through extraction method five (99.8 % recovery after optimization).

Yohimbe is reported as containing up to as much as 6 % yohimbine alkaloids. Although yohimbine is the main active compound, there have been few studies of the expected percent composition of this in the total bark. A commercial product may therefore claim to be providing several hundred milligrams of yohimbe bark, without a traceable level of yohimbine present. This was displayed by several of the samples in question (Table 15).

This method proved to be useful for a broad range of product concentrations. Several products, including C1, T2, T5 and L2 demonstrated no detectable yohimbine. The most potent product was C4 (Yohimbe Fuel by Twinlab), displaying 1.61 % yohimbine (percent by weight) against a claim for 2 % yohimbine per capsule.

Summary.

The use of a fractional factorial design toward this investigation was beneficial for a number of reasons. First, the number of experimental runs that had to be completed in order to attain this bulk of information was far less than a full design would have afforded. Second, a large variation in experimental parameters was evaluated, gaining valuable trend information. This broad range may not have been covered had the one

factor at a time approach been utilized. Through this factorial design, it was possible to determine the experimental conditions that would lead to an optimized separation without disregarding the effects at extreme setpoints. And finally, interaction effects were calculated and interpreted in method optimization. These effects are not evaluated using the traditional one factor at a time method.

Future Work.

Work in addition to this study could focus on the use of a mass spectrometer (MS) in order to characterize the peaks that co-migrated with the peak characteristic to yohimbine. An online CE-MS with an electrospray injection would allow for a second dimension to be added to this method. The eserine and yohimbine peaks, as well as the unknown peaks would be characterized and identified simultaneously while maintaining a minute quantity of generated waste.

A focus on biological fluids could be completed. This effort would require the development of an extraction of yohimbine from the biological fluid of interest, and employ the same CE methods utilized in this investigation. An extraction of this type would prove useful for a number of relevant studies.

An investigation could focus on the clearance time of this compound from the human body, including the percentage that is removed from the body unchanged. This work may lead to an investigation of the therapeutic amounts of yohimbine needed in the human body. Results from a study of this magnitude could prove useful in the regulation of the product by the Federal government.

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APPENDIX.

Table 18. Complete results from factorial design one.

Run #	MigTime (1)	MigTime (2)	Area (1)	Area (2)	Symmetry (1)	Symmetry (2)	Plates (1)	Plates (2)	Resolution	Selectivity
1	37.4	44.0	4.5	38.8	0.04	0.26	89518	1790	2.82	1.18
2	6.3	7.6	20.3	63.9	0.43	0.32	78063	36031	10.06	1.20
3	3.4	4.3	10.4	68.0	0.35	0.37	84571	54102	15.3	1.27
4	33.8	45.7	15.6	54.2	0.48	0.31	199267	118471	28.43	1.35
5	5.8	6.7	27.1	62.9	0.09	0.04	9334	9395	3.25	1.14
6	37.1	42.2	28.7	54.1	0.11	0.01	52673	42974	7.31	1.14
7	21.3	24.1	12.0	30.1	0.09	0.12	45669	53199	6.93	1.13
8	2.5	2.8	13.4	31.2	0.22	0.07	6441	8037	2.71	1.14
9	1.5	1.6	8.1	51.5	0.07	0.02	1783	161	0.28	1.06
10	11.5	12.2	23.7	58.8	0.01	0.02	1628	1651	0.57	1.06
11	7.3	7.7	11.3	24.4	0.02	0.00	1545	1760	0.45	1.05
12	0.7	0.7	3.8	25.7	0.18	0.05	1922	139	0.18	1.04
13	22.2	24.2	24.8	28.8	0.22	0.40	189953	285267	10.29	1.09
14	2.3	2.5	24.8	29.4	0.83	0.16	12733	9294	2.01	1.08
15	1.2	1.3	10.9	11.8	0.12	0.78	44260	24703	4.06	1.10
16	9.3	10.2	14.8	18.4	0.14	0.29	192668	349542	10.76	1.09
17	36.0	42.4	20.4	41.5	0.13	1.13	34030	3705	3.61	1.18
18	5.5	6.3	17.9	42.7	5.00	0.53	29523	92268	7.13	1.14
19	3.0	3.4	8.3	20.4	5.21	0.29	41379	91817	7.78	1.13
20	18.7	21.4	6.3	21.0	3.21	0.59	184280	245394	15.56	1.14
21	8.1	9.6	24.1	52.8	0.29	0.21	69341	44424	9.45	1.18
22	51.1	59.9	12.8	24.2	0.49	0.27	153044	110945	14.15	1.17
23	30.2	35.2	4.9	13.5	2.81	0.27	212205	182835	17.68	1.17
24	3.0	3.5	8.1	17.9	0.23	0.21	119842	68168	11.29	1.17
25	4.6	5.0	10.2	24.6	0.24	0.29	69766	70182	5.99	1.10
26	24.5	29.2	8.3	13.0	0.94	0.79	149374	72380	13.59	1.19
27	14.0	16.6	7.4	6.4	0.51	0.68	197095	87767	14.75	1.18
28	1.8	1.9	3.7	9.4	0.41	0.29	84845	96277	6.47	1.09
29	14.0	14.8	3.1	8.5	0.10	0.31	128866	343645	6.67	1.06
30	1.4	1.5	3.7	17.2	0.12	0.15	39219	80385	2.72	1.05
31	0.8	0.8	1.5	8.3	0.25	0.19	48342	87909	3.02	1.05
32	5.9	6.3	1.9	5.8	0.11	0.25	151956	371845	6.39	1.05
33	46.0	52.5	98.7	254.0	3.91	0.34	45323	120367	8.80	1.14
34	5.8	6.5	117.8	240.4	22.20	9.53	1471	6868	1.58	1.13
35	3.1	3.4	49.3	104.4	17.91	5.31	2775	13355	2.10	1.12
36	17.6	19.8	41.0	112.9	9.57	2.16	30344	126440	6.98	1.12
37	8.0	9.4	113.9	219.0	0.13	0.08	20531	13382	5.10	1.18
38	51.2	59.9	92.1	167.2	0.52	0.25	97078	74261	11.35	1.17
39	30.2	35.4	55.9	106.1	0.25	0.19	128489	96391	13.07	1.17
40	2.9	3.4	43.0	80.7	0.13	0.08	16882	16019	4.86	1.16
41	4.4	4.8	100.2	203.2	0.26	0.09	1177	9566	1.22	1.1
42	30.8	33.8	150.4	263.5	0.15	0.14	39597	45897	4.74	1.1
43	19.7	21.7	57.8	113.7	0.11	0.12	54173	68701	6.02	1.1

44	1.7	1.9	43.4	96.9	0.08	0.15	1005	6138	1.19	1.11
45	13.2	14.3	96.0	313.5	0.03	0.01	1699	7032	1.02	1.08
46	1.2	1.3	52.5	344.4	0.09	0.03	1682	270	0.34	1.06
47	0.7	0.8	27.5	152.9	0.10	0.04	1336	279	0.36	1.07
48	5.6	6.0	48.9	169.7	0.08	0.13	1576	8156	1.00	1.07
49	37.0	43.9	33.0	118.3	0.21	0.76	119.56	25.19	2.84	1.19
50	8.6	11.3	35.7	174.9	0.32	0.14	42503	14007	9.69	1.31
51	4.2	5.4	15.3	73.0	0.20	0.09	61349	19712	11.29	1.30
52	33.8	45.5	25.9	125.1	0.17	0.003	155309	71466	22.93	1.35
53	5.9	6.7	34.7	90.3	0.04	0.05	7037	6462	2.72	1.14
54	35.9	40.8	24.5	64.2	0.12	0.14	51546	44628	6.87	1.13
55	20.9	23.7	14.4	38.6	0.24	0.15	43655	47312	6.64	1.13
56	2.5	2.9	16.2	42.1	0.09	0.04	6820	12209	3.03	1.14
57	1.6	1.7	6.6	27.7	0.07	0.02	1541	304	0.33	1.06
58	12.4	13.1	12.4	23.7	0.02	0.04	1528	3614	0.72	1.06
59	7.4	7.8	5.6	12.2	0.11	0.02	2167	5300	0.80	1.06
60	0.7	0.7	3.0	13.4	0.14	0.00	1706	298	0.32	1.06
61	22.3	24.2	14.3	14.3	0.50	0.77	261346	341994	11.53	1.09
62	2.4	2.6	11.7	14.0	0.23	0.53	51961	32328	4.26	1.09
63	1.2	1.3	5.2	5.0	0.20	0.42	188263	104210	7.86	1.09
64	9.3	10.1	7.1	8.3	0.28	0.41	358699	540072	13.89	1.09
65	16.0	19.2	67.9	120.1	0.11	0.12	29908	24734	7.36	1.20
66	16.2	19.4	26.8	55.1	0.29	0.25	70069	40162	10.18	1.20
67	8.9	9.6	51.8	79.1	0.11	0.12	44851	45231	4.08	1.08
68	9.0	9.8	13.5	24.2	0.27	0.25	131267	131855	7.00	1.08

Table 19. Complete results factorial design two.

Run #	Mig Time (1)	Mig Time (2)	Area (1)	Area (2)	Symmetry (1)	Symmetry (2)	Plates (1)	Plates (2)	Resolution	Selectivity
1	4.00	4.51	20.1	141.0	0.10	0.03	3375	3905	1.79	1.13
2	3.35	3.68	17.8	131.6	0.13	0.04	2577	4103	1.31	1.10
3	1.89	2.12	8.3	68.0	0.05	0.05	2614	4513	1.59	1.12
4	1.61	1.77	8.2	67.7	0.12	0.06	1146	4506	1.08	1.10
5	6.45	7.85	34.0	243.7	0.14	0.11	14643	14366	5.90	1.22
6	5.77	6.70	19.5	164.7	0.12	0.12	33222	30660	6.61	1.16
7	2.95	3.56	13.0	103.3	0.14	0.08	19311	19246	6.49	1.21
8	2.57	2.95	9.1	78.8	0.15	0.12	34762	32197	6.38	1.15
9	3.03	3.42	13.1	113.9	0.10	0.05	11032	14038	3.35	1.13
10	3.46	4.00	17.1	115.0	0.15	0.05	8112	11021	3.50	1.16

Figure 16. Two factor interactions on resolution: factorial design one (fractional)

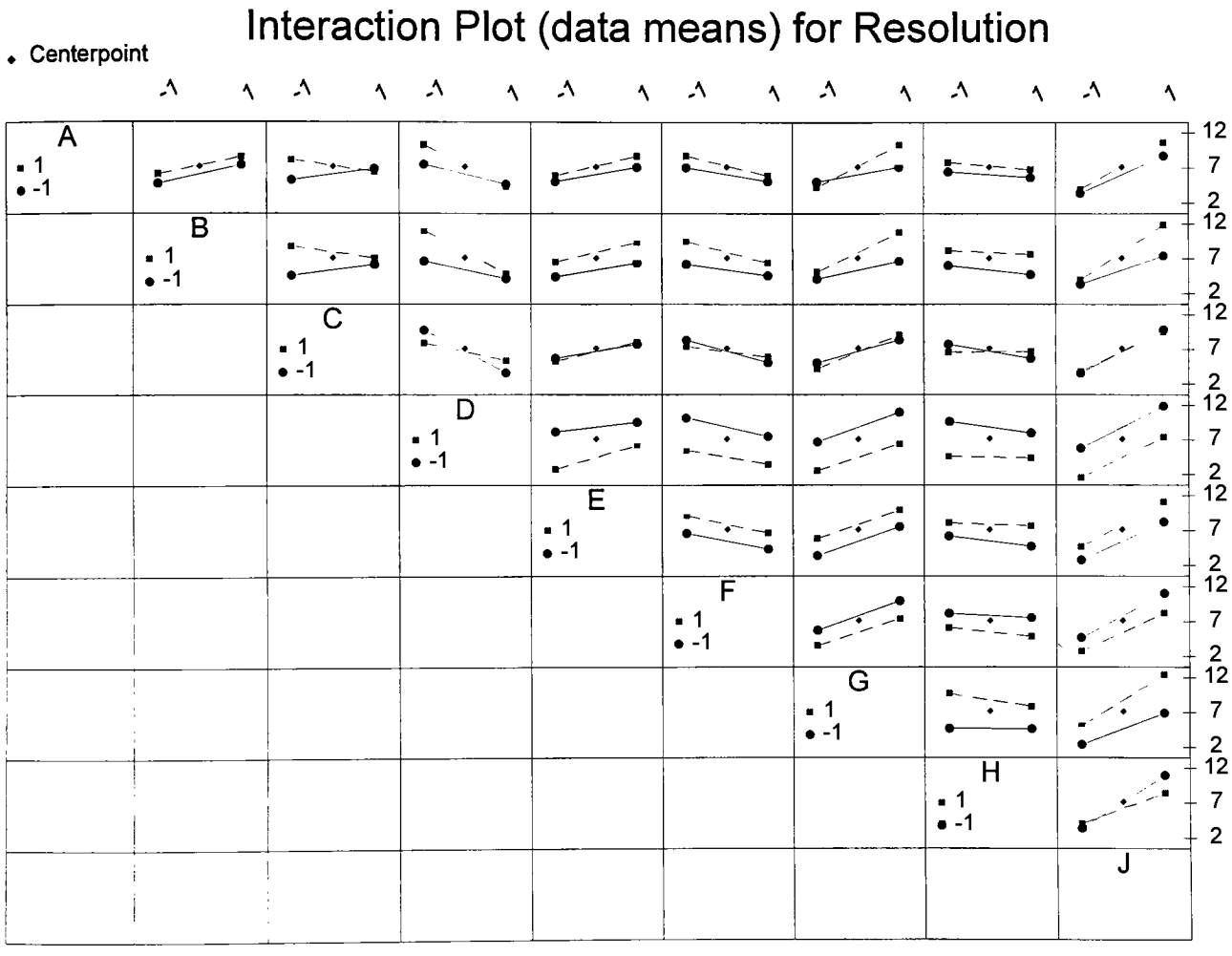
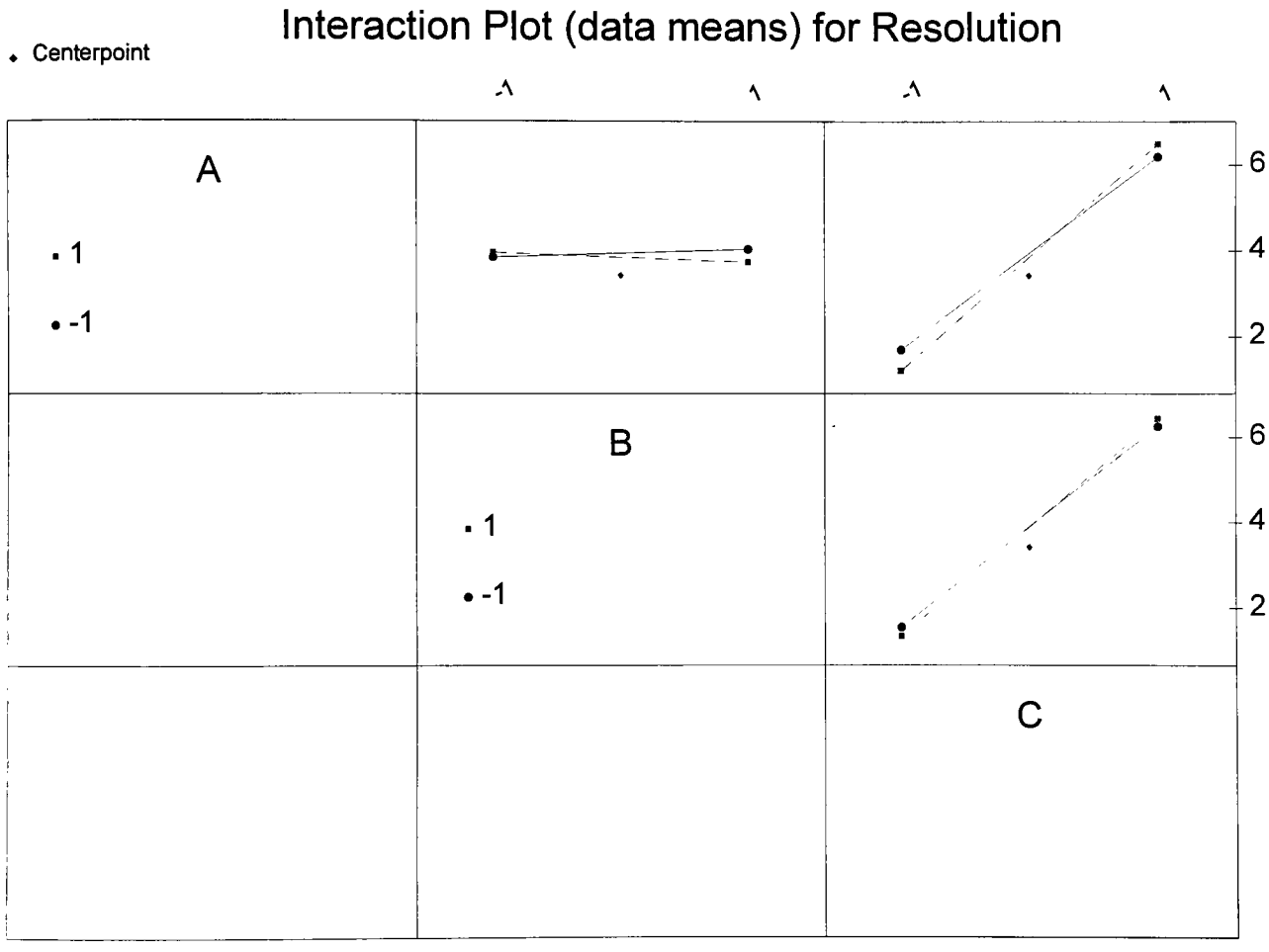
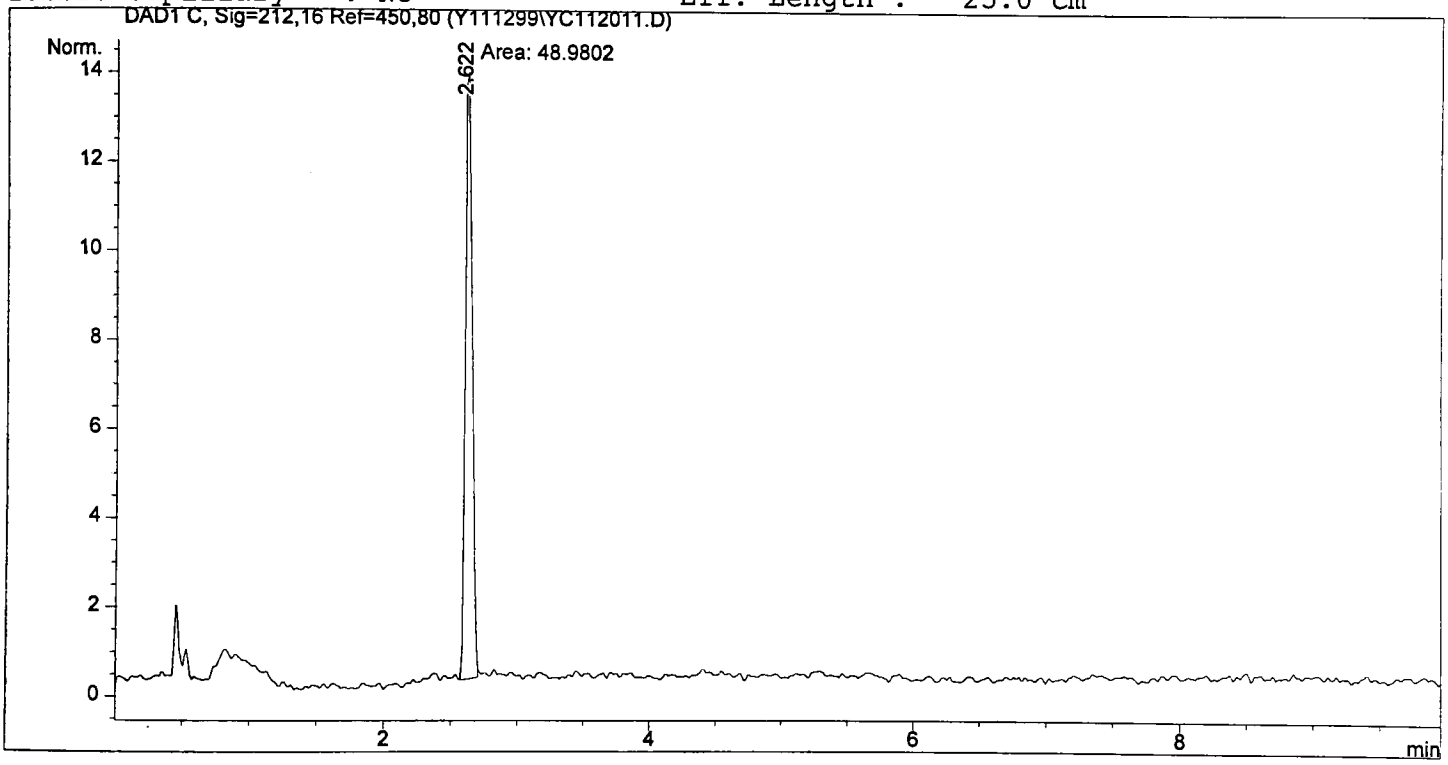


Figure 17. Two factor interactions on resolution: factorial design two (full)



```
==========================================================================
Injection Date  : 11/15/99 2:51:32 AM          Seq. Line :  11
Sample Name     : c1                               Vial :  18
Acq. Operator   : Jonathan Cooper                   Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 1:06:13 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper


==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length     :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```

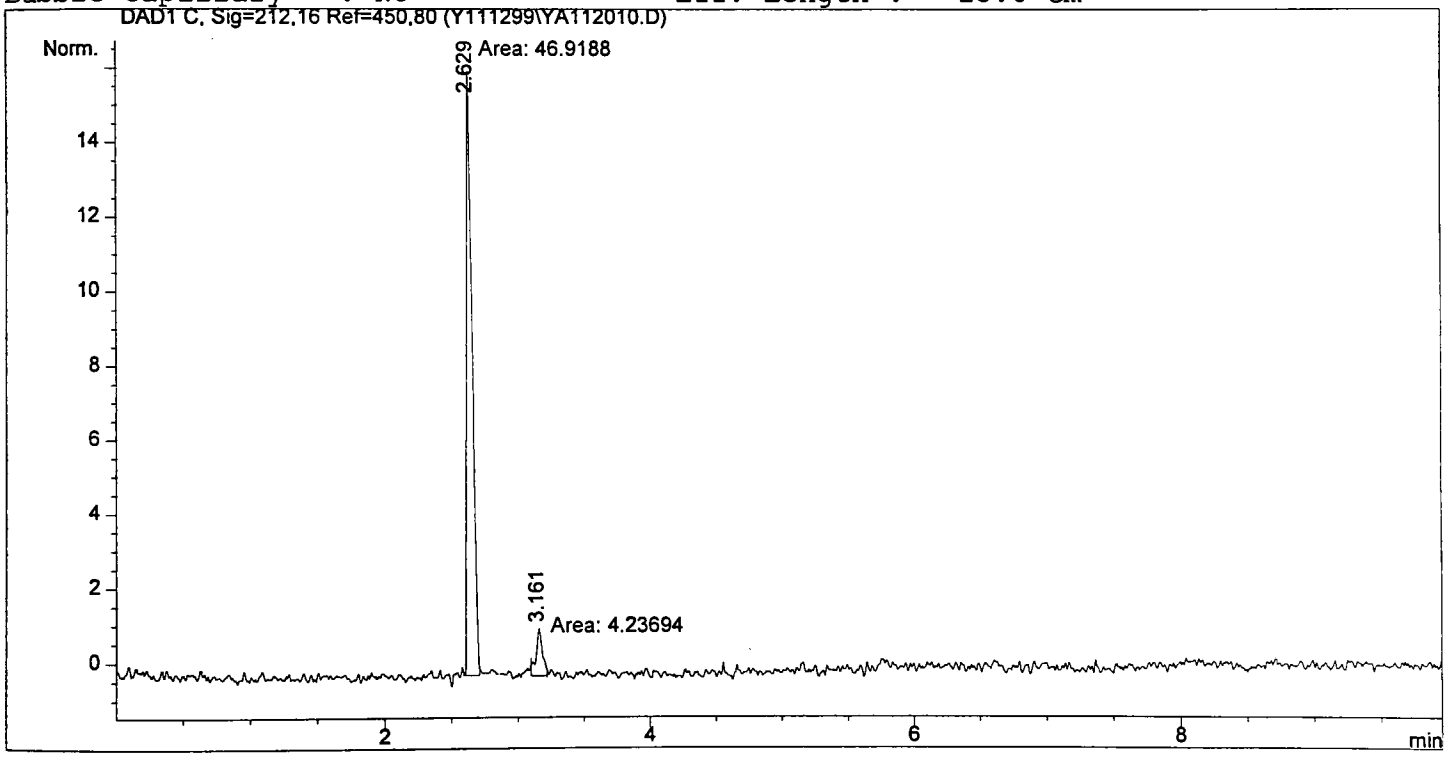



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000


Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.622	-	48.98016	14.02603	0.55	0.0592	10876	-	-

*** End of Report ***

```
==========================================================================
Injection Date  : 11/13/99 5:05:34 PM          Seq. Line :  10
Sample Name     : c2                                Vial :  19
Acq. Operator   : Jonathan Cooper                    Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/13/99 4:46:17 PM by Jonathan Cooper
                  (modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 7:59:24 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                      Batch#:
Diameter           : 25.0 µm              Length     :   33.5 cm
Bubble capillary   : No                Eff. Length :   25.0 cm
```

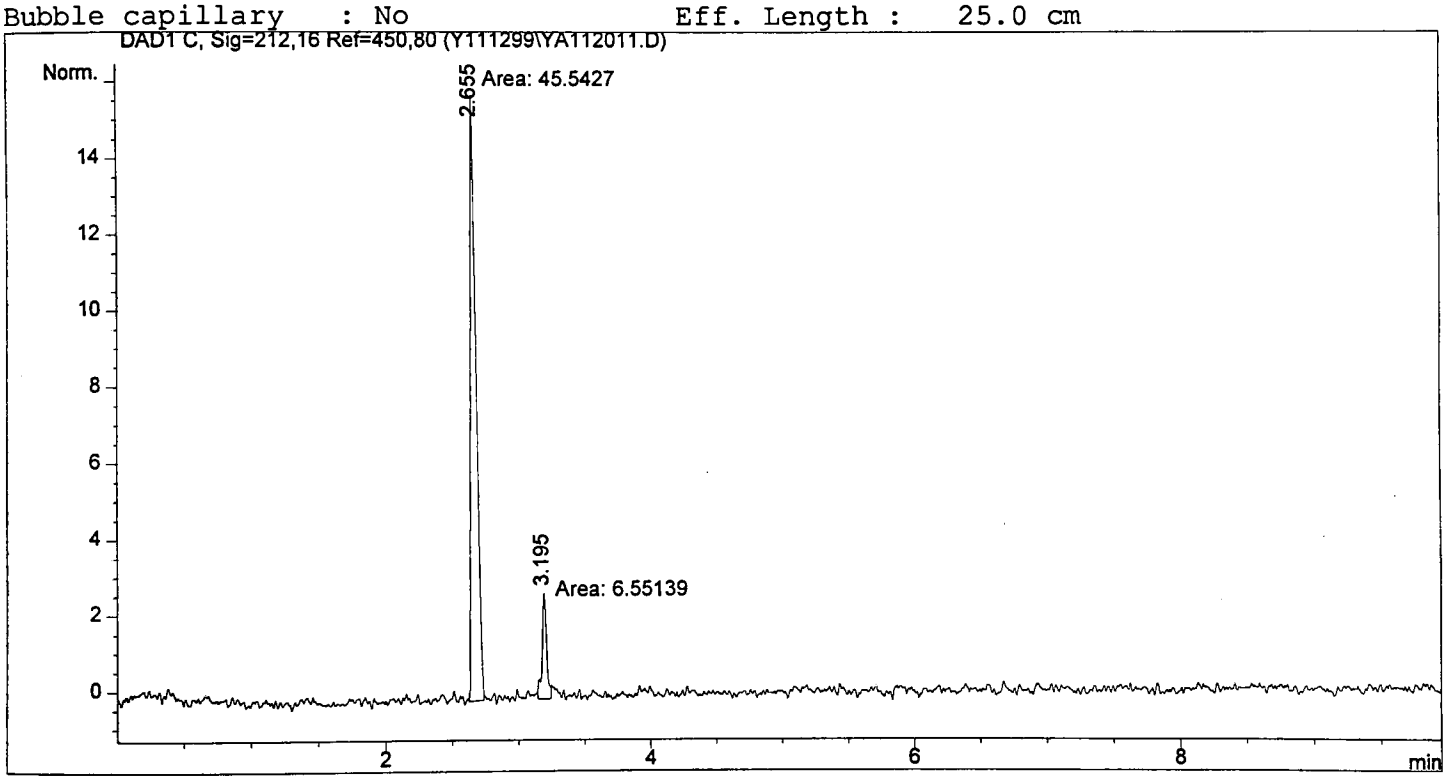



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.629	-	46.91885	16.78396	0.33	0.0455	18536	-	-
3.161	-	4.23694	1.27554	0.95	0.0444	28019	6.95	1.20

*** End of Report ***

```
==========================================================================
Injection Date  : 11/13/99 5:35:07 PM          Seq. Line :  11
Sample Name     : c3                                Vial :  20
Acq. Operator   : Jonathan Cooper                    Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/13/99 5:15:47 PM by Jonathan Cooper
                  (modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 8:00:50 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                      Batch#:
Diameter           : 25.0 µm              Length     :   33.5 cm
Bubble capillary   : No                Eff. Length :   25.0 cm
```




```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.655	-	45.54269	16.28253	0.34	0.0455	18906	-	-
3.195	-	6.55139	2.82070	0.87	0.0344	47899	7.94	1.20

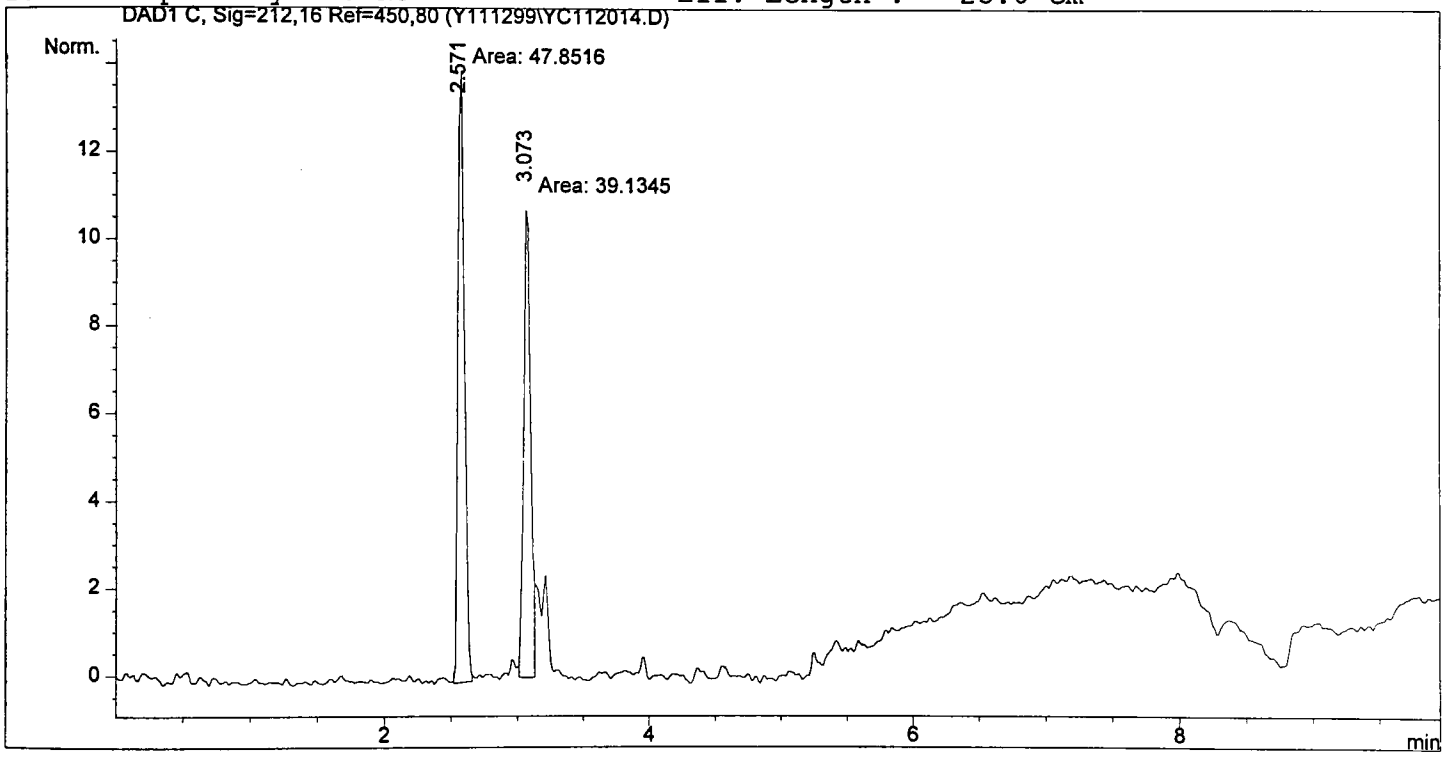
*** End of Report ***

==========

Injection Date : 11/15/99 4:07:49 AM Seq. Line : 14
Sample Name : c4 Vial : 21
Acq. Operator : Jonathan Cooper Inj : 1
Acq. Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/15/99 1:05:21 PM by Jonathan Cooper
(modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========

Capillary : Barefused Silica
Product# : Batch#:
Diameter : 25.0 µm Length : 33.5 cm
Bubble capillary : No Eff. Length : 25.0 cm



==========

Area Percent Report with Performance

==========

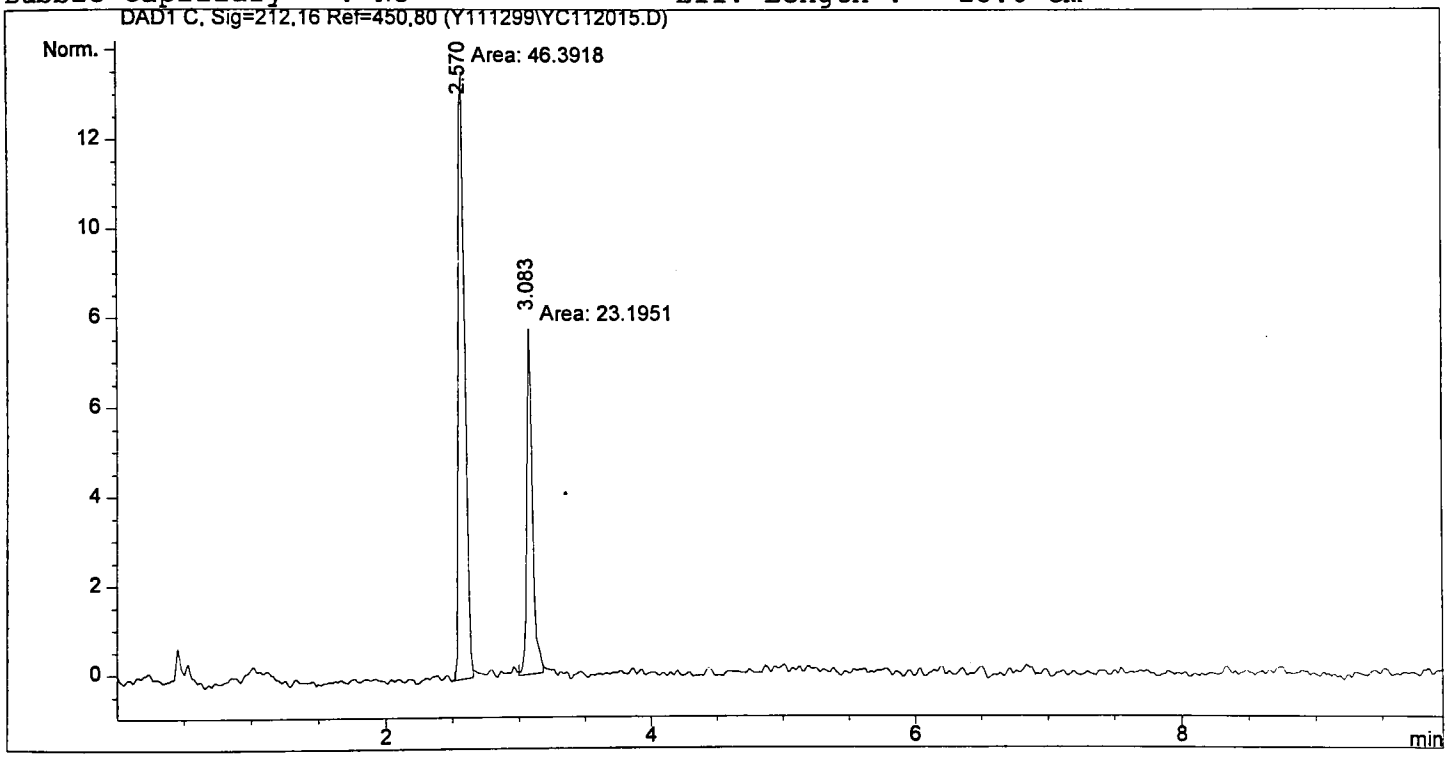
Area Calculation Mode : Measured Area
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
Results obtained with enhanced integrator!

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.571	-	47.85159	14.28505	0.61	0.0580	10871	-	-
3.073	-	39.13450	11.21555	0.74	0.0612	13975	4.94	1.20

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 4:33:15 AM          Seq. Line :  15
Sample Name     : c5                                Vial :  22
Acq. Operator   : Jonathan Cooper                    Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 1:03:39 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                      Batch#:
Diameter           : 25.0 µm              Length     :   33.5 cm
Bubble capillary   : No                Eff. Length :   25.0 cm
```

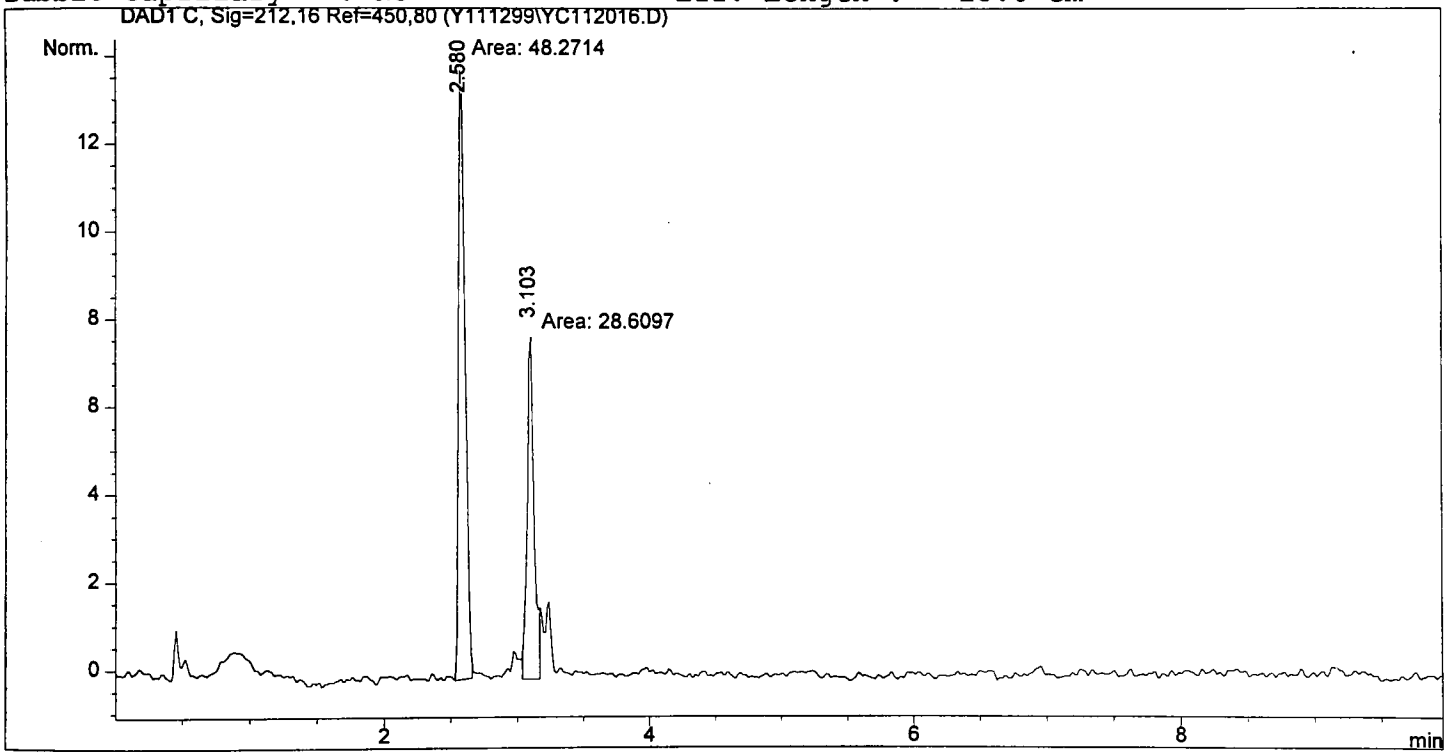



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.570	-	46.39185	13.94060	0.61	0.0573	11163	-	-
3.083	-	23.19514	8.03220	0.75	0.0491	21824	5.67	1.20

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 4:58:42 AM          Seq. Line :  16
Sample Name     : c6                                Vial :  23
Acq. Operator   : Jonathan Cooper                    Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 1:02:35 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                      Batch#:
Diameter           : 25.0 µm              Length    :   33.5 cm
Bubble capillary   : No                Eff. Length :   25.0 cm
```

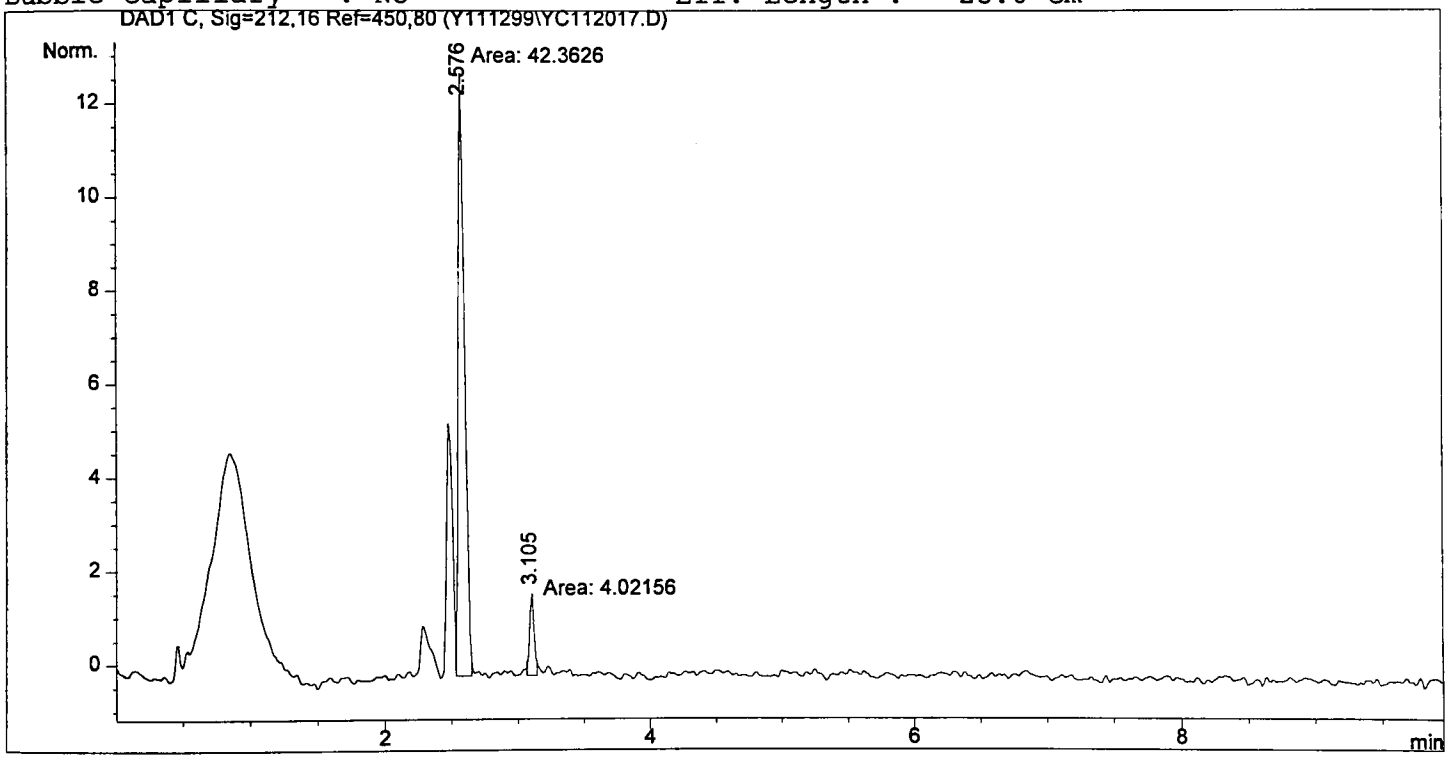



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000


Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.580	-	48.27141	14.88949	0.53	0.0557	11889	-	-
3.103	-	28.60966	8.10988	0.84	0.0583	15672	5.39	1.20

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 5:24:08 AM          Seq. Line :  17
Sample Name     : c7                               Vial :  24
Acq. Operator   : Jonathan Cooper                   Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 1:01:58 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length    :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```



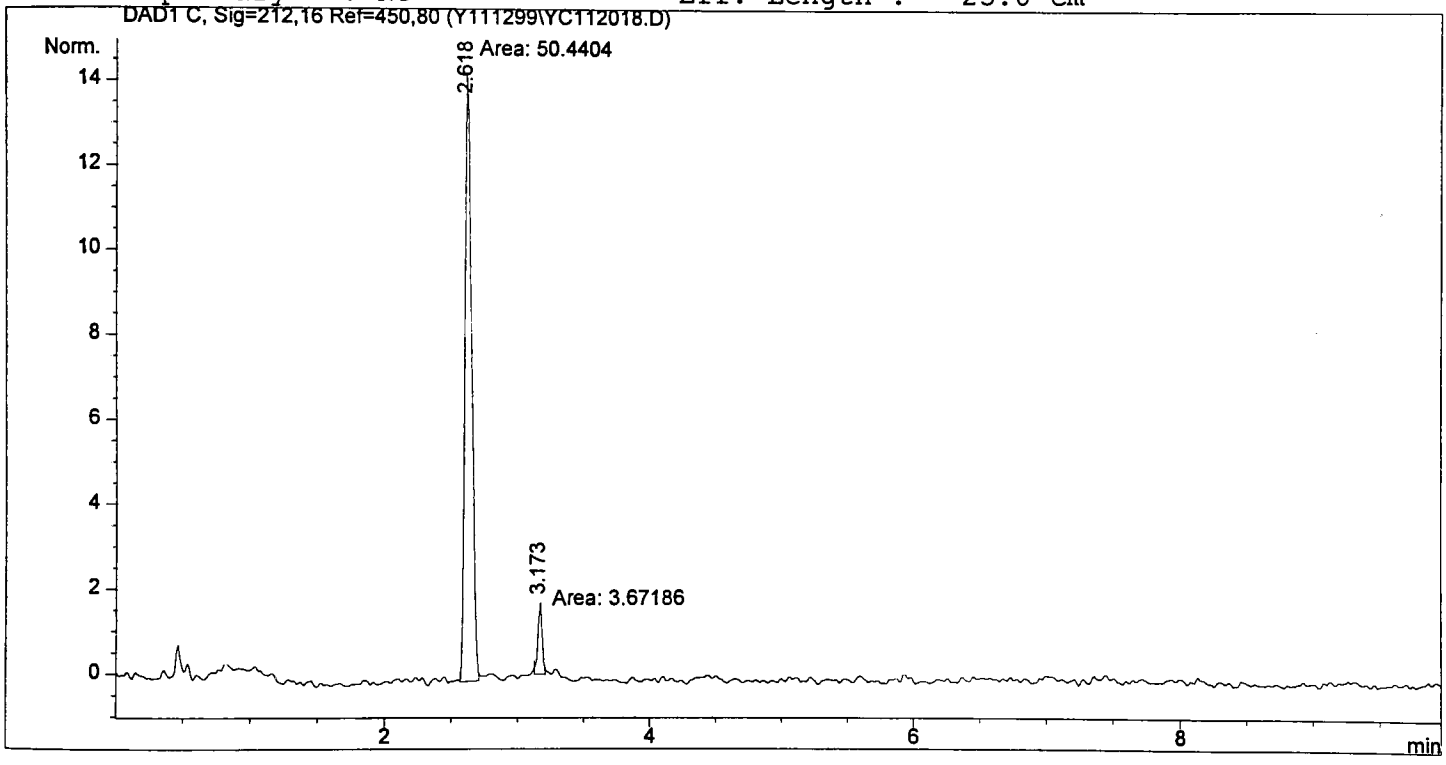

```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.576	-	42.36260	13.20951	0.63	0.0557	11855	-	-
3.105	-	4.02156	1.80595	0.85	0.0435	28260	6.27	1.21

==========

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 5:49:31 AM          Seq. Line :  18
Sample Name     : t1                               Vial :  25
Acq. Operator   : Jonathan Cooper                   Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 1:01:10 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper


==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length    :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```

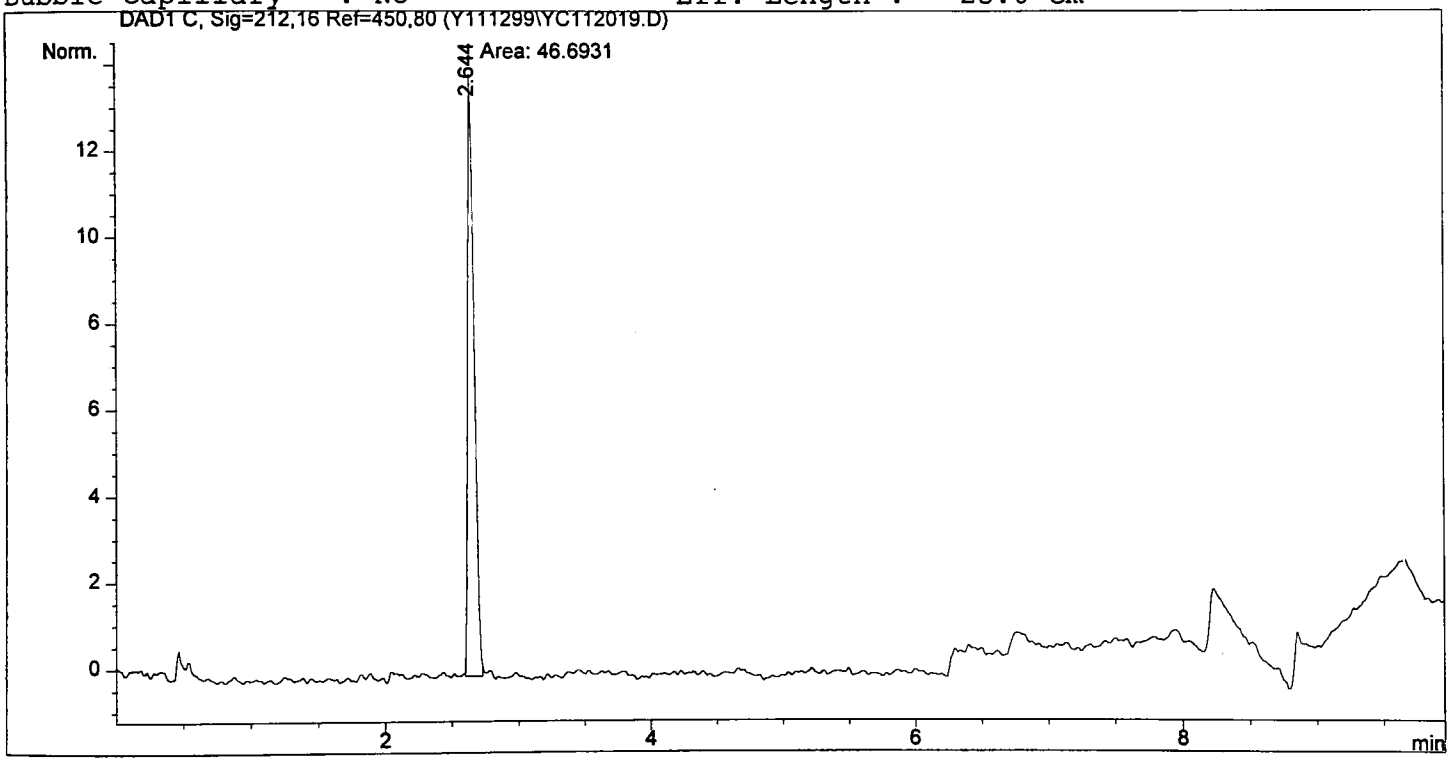



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000


Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.618	-	50.44037	15.12113	0.54	0.0565	11910	-	-
3.173	-	3.67186	1.73705	1.03	0.0378	39075	6.91	1.21

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 6:15:00 AM          Seq. Line :  19
Sample Name     : t2                             Vial :  26
Acq. Operator   : Jonathan Cooper                 Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 12:59:47 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length    :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```

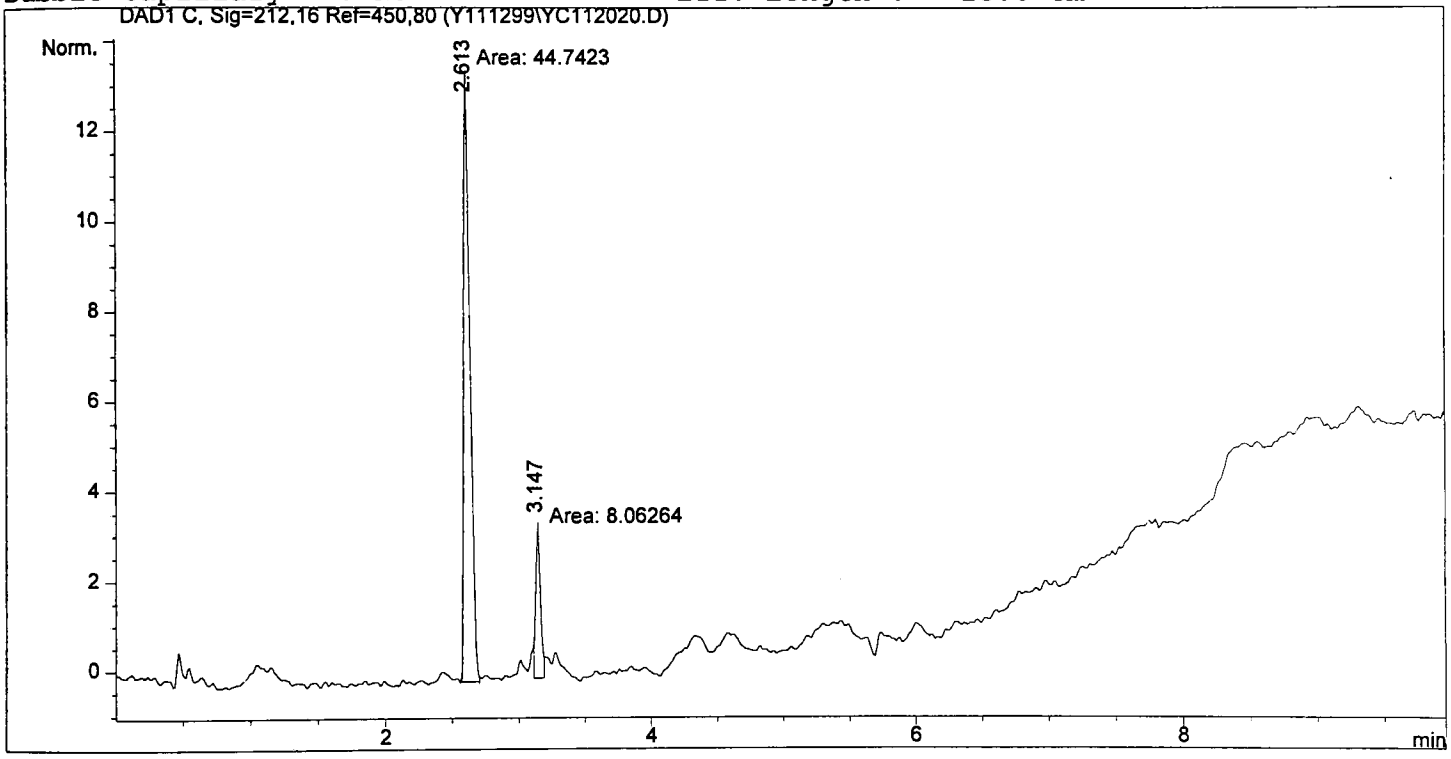



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.644	-	46.89314	14.44299	0.57	0.0557	12485	-	-

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 6:40:24 AM          Seq. Line :  20
Sample Name     : t3                              Vial :  27
Acq. Operator   : Jonathan Cooper                  Inj :   1
Method          : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length    :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```




```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

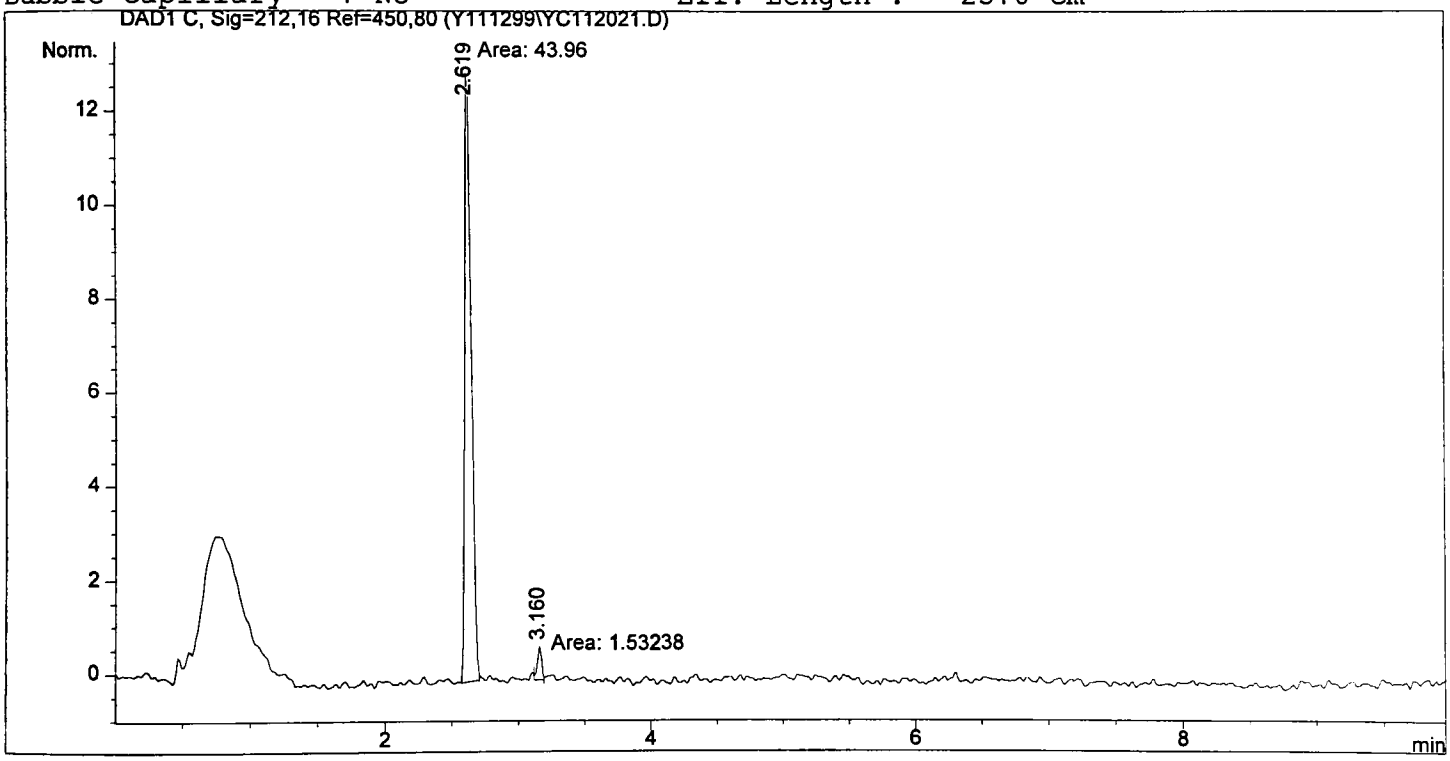
MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.613	-	44.74234	13.83238	0.63	0.0557	12202	-	-
3.147	-	8.06264	3.55629	0.80	0.0470	24879	6.11	1.20

==========

Injection Date : 11/15/99 7:05:51 AM Seq. Line : 21
Sample Name : t4 Vial : 28
Acq. Operator : Jonathan Cooper Inj : 1
Acq. Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/15/99 12:56:05 PM by Jonathan Cooper
(modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========

Capillary : Barefused Silica
Product# : Batch#:
Diameter : 25.0 µm Length : 33.5 cm
Bubble capillary : No Eff. Length : 25.0 cm



==========

Area Percent Report with Performance

==========

Area Calculation Mode : Measured Area
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
Results obtained with enhanced integrator!

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.619	-	43.96003	13.84906	0.55	0.0557	12257	-	-
3.160	-	1.53238	7.32475e-1	1.05	0.0417	31872	6.53	1.21

==========

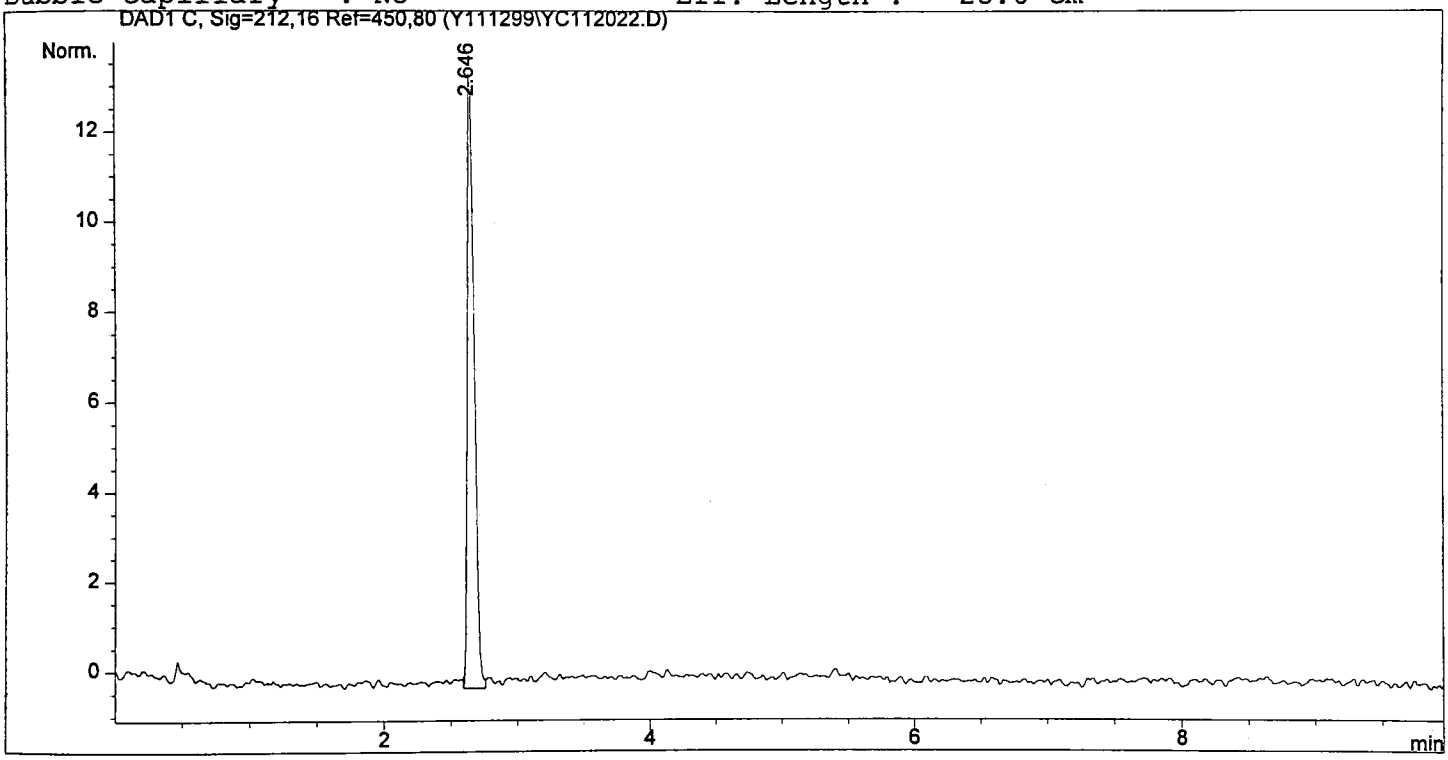
*** End of Report ***

==========

Injection Date : 11/15/99 7:31:22 AM Seq. Line : 22
Sample Name : t5 Vial : 29
Acq. Operator : Jonathan Cooper Inj : 1
Acq. Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed : 11/15/99 12:56:53 PM by Jonathan Cooper
(modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========

Capillary : Barefused Silica
Product# : Batch#:
Diameter : 25.0 µm Length : 33.5 cm
Bubble capillary : No Eff. Length : 25.0 cm



==========

Area Percent Report with Performance

==========

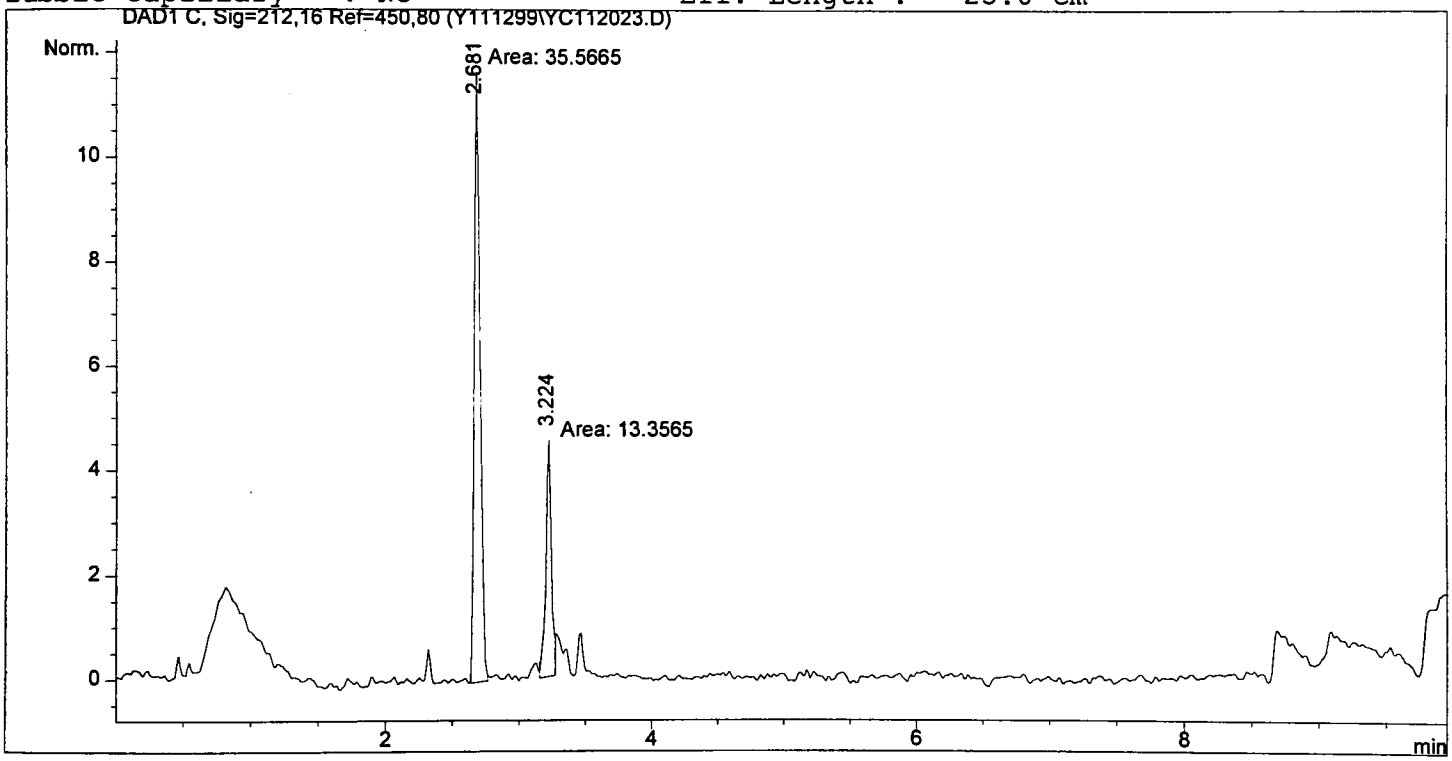
Area Calculation Mode : Measured Area
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
Results obtained with enhanced integrator!

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.646	-	48.72402	14.28163	0.52	0.0578	11620	-	-

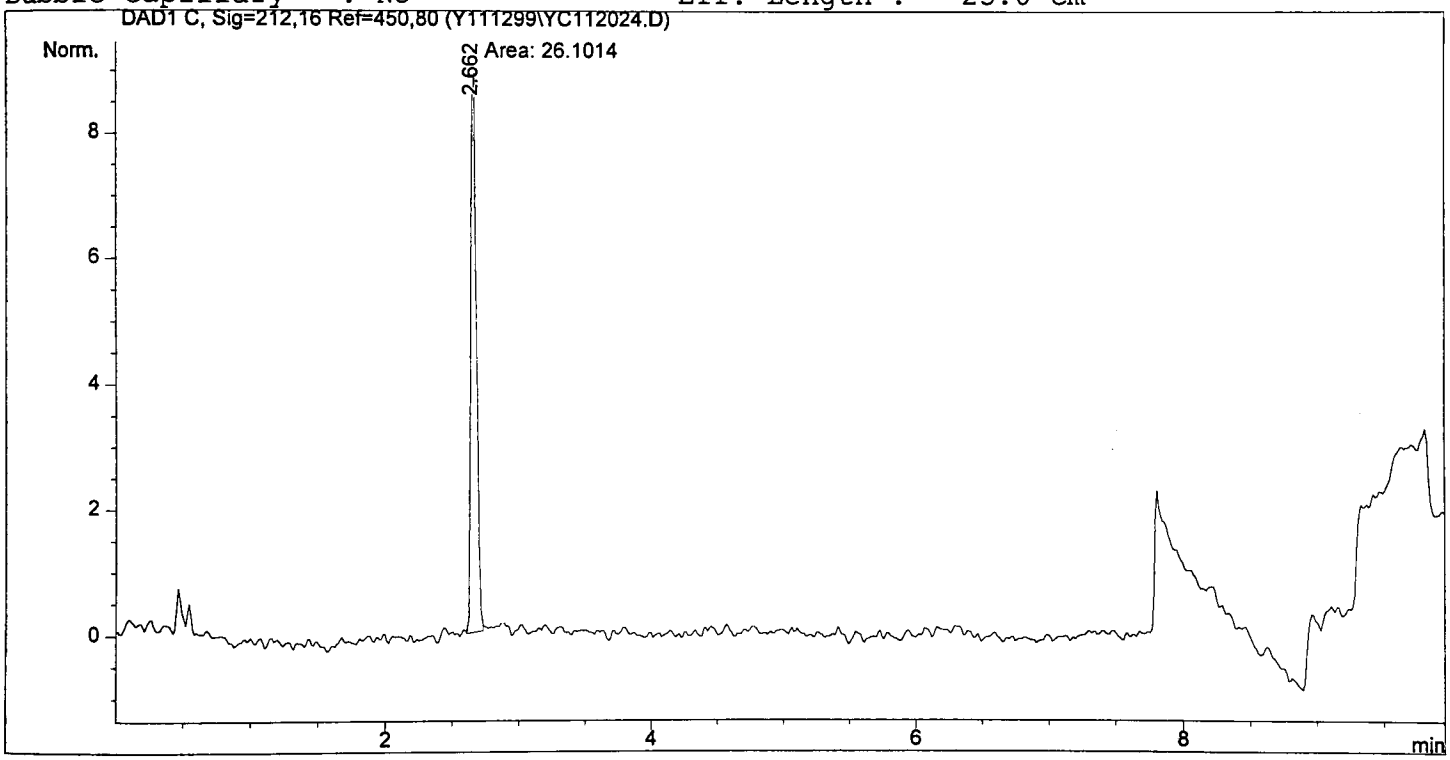
*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 7:57:53 AM          Seq. Line :  23
Sample Name     : L1                              Vial :  30
Acq. Operator   : Jonathan Cooper                  Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 12:57:24 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length     :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```




```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

```
==========================================================================
Injection Date  : 11/15/99 8:23:23 AM          Seq. Line :  24
Sample Name     : L2                               Vial :  31
Acq. Operator   : Jonathan Cooper                   Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 12:58:40 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length     :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```

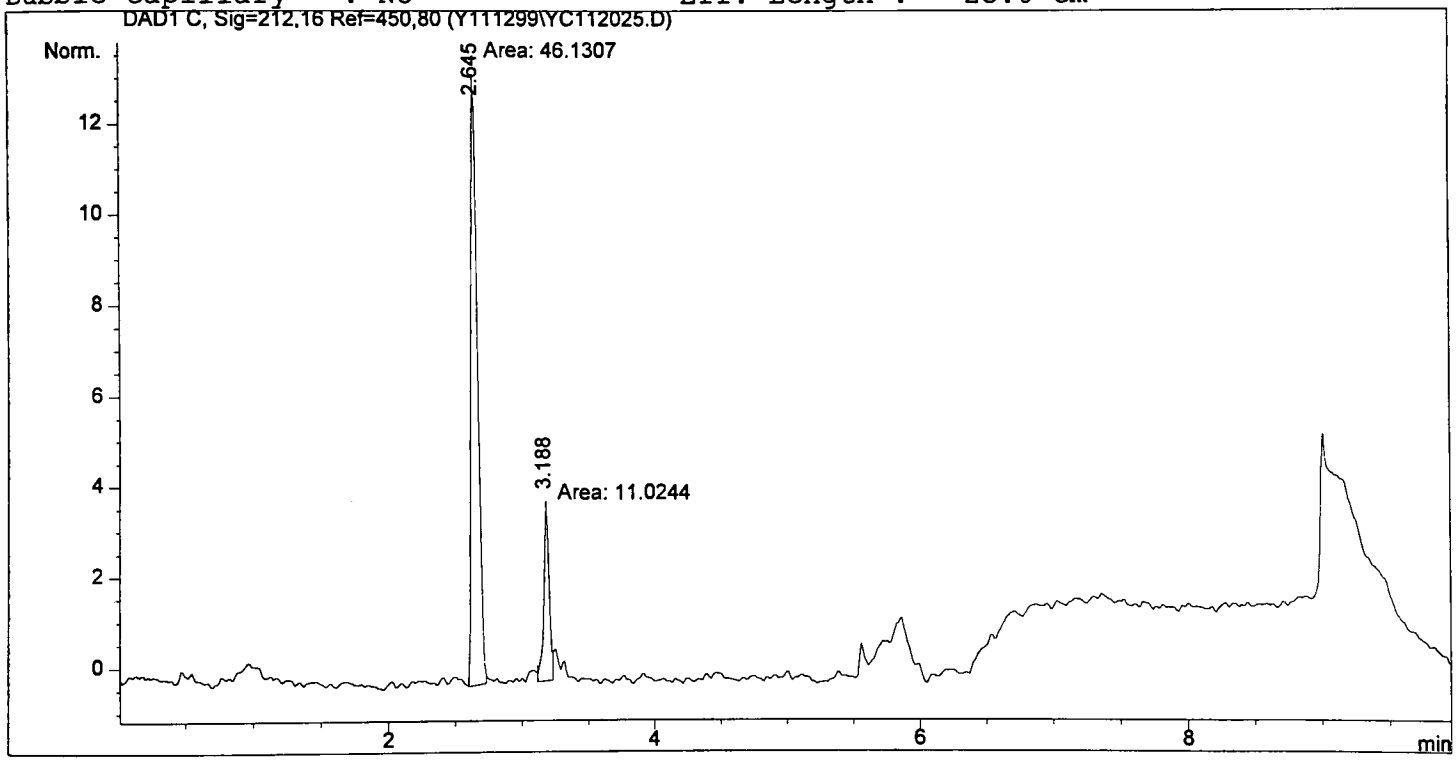



```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000


Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.662	-	26.10143	9.27425	0.70	0.0507	15292	-	-

*** End of Report ***

```
==========================================================================
Injection Date  : 11/15/99 8:48:41 AM          Seq. Line :  25
Sample Name     : ybp                              Vial :  32
Acq. Operator   : Jonathan Cooper                   Inj :   1
Acq. Method     : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/14/99 10:20:27 PM by Jonathan Cooper
Analysis Method : C:\HPCHEM\1\METHODS\CEP\YOHIMBIN.M
Last changed    : 11/15/99 12:59:10 PM by Jonathan Cooper
                  (modified after loading)
Yohimbine Method Development
Jonathan Cooper

==========================================================================

Capillary          :  Barefused Silica
Product#           :                    Batch#:
Diameter           : 25.0 µm            Length     :   33.5 cm
Bubble capillary   : No               Eff. Length :   25.0 cm
```




```
==========================================================================
                Area Percent Report with Performance
==========================================================================

Area Calculation Mode :      Measured Area
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: DAD1 C, Sig=212,16 Ref=450,80
 Results obtained with enhanced integrator!
```

MigTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.645	-	46.13070	14.28615	0.56	0.0549	12854	-	-
3.188	-	11.02444	4.10314	0.99	0.0480	24442	6.21	1.21

==========

*** End of Report ***